Bacterial diversity and its interaction with metals in glacio-marine environment of Arctic

A Thesis submitted in partial fulfilment for the Degree of

DOCTOR OF PHILOSOPHY

in the School of Earth, Ocean and Atmospheric Sciences

Goa University

By

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April 2022

DECLARATION

I, Ms. Femi Anna Thomas hereby declare that this thesis represents work which has been carried out by me and that it has not been submitted, either in part or full, to any other University or Institution for the award of any research degree.

Place: Taleigao Plateau.

Date: 13-04-2022

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CERTIFICATE

I hereby certify that the above Declaration of the candidate, Ms. Femi Anna Thomas is true and the work was carried out under my supervision.

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Acknowledgement

First and foremost, I express my heartfelt gratitude to my guide Late Dr. K. P. Krishnan, Scientist-E and Group Director, Arctic Operations, NCPOR-India for the valuable guidance and encouragement throughout the period of my research work. With the insistence on fundamentals of research work, I was also always provided with an affable environment at his lab which enabled me to enjoy my work. All the encouragement and support are deeply acknowledged.

My Sincere thanks to the Ministry of Earth Sciences, India and Goa University for providing the funding and research administrative facilities.

Many thanks to Dr. M. Ravichandran, former Director, NCPOR and Secretary, MoES, for his support and DRC members Prof. G. N. Nayak and Prof. Savita Kerkar for their constructive suggestions and feedback in all the DRC meetings which had improved my research.

With deep gratitude, I would like to acknowledge the unwavering support and guidance provided by Dr. Thamban Meloth, Director of the National Centre for Polar and Ocean Research (NCPOR), and Dr. Manish Tiwari, Group Director, Arctic Division, throughout the administrative procedures associated with my PhD. I gratefully acknowledge Dr. Rupesh Kumar Sinha, Dr. Anand Jain, Dr. Jabir. T, Dr. Vipindas P. V, and Dr. Venkatachalam Siddharthan for all their support and guidance throughout my research life at NCPOR. Their continued guidance in the lab works and data analysis helped me immensely to successfully complete my research. I extend my deep gratitude to Dr. Anand Jain and Ms. Archana Singh who were with me in the Indian Arctic Expedition 2017 and 2018 and helped me at all stages of the expedition right from the sampling and experimental setup. Their constant support and love had made my Arctic Expeditions the most memorable experience I ever had in my PhD life. I would also like to thank Ms. Nazira Begum and Mrs. Soumya Tari for all the technical help provided. All members of my Arctic Team at NCPOR have helped me throughout my ups and downs and encouraged me immensely in my research work.

I am thankful to Dr. Thamban Meloth, Dr. Manish Tiwari, Dr. P.V.Bhaskar for providing me with the instrument support and lab facilities. I would also extend my

gratitude to Divya David, Vikash Kumar, Viola, Lathika P, Ajit Singh, Lavkush Patel, Gautami Samui, Melena Soares and Amit Sarkar for all their help with different analyses for my research work.

I acknowledge my profound gratitude to my alma-mater CUSAT and to all my Professors especially Prof. A. A. Mohamed Hatha, and Prof. Rosamma Philip, for all their love and encouragement from the MSc time. They were my real motivation to pursue my research career in Microbiology.

My sincere thanks to Dr. Mahesh Mohan, Assistant Professor, School of Environmental Sciences, Mahatma Gandhi University and his students Dr. Gopikrishnan, Dr. Kannan, Binish, John for all their support during my analysis time at MG University in 2018. I also extend my thanks to Dr. Sajeevan T. P, Associate Professor, National Centre for Aquatic Animal Health, CUSAT for all his help in my research.

I would like to thank all my hostel mates right from the first year at NCPOR- Parul, Girijakalyani, Aritri, Gautami, Anvita, Gayatri and Anju. They have made my NCPOR life happier and memorable. I would also like to extend my thanks to all my colleagues- fellow PhD students and Scientists at NCPOR who were always open to helping me whenever required and for all the fun-filled days.

I wish to thank all administrative staff at NCPOR for all their timely help and technical support in all stages of my research.

I am also thankful to my friends Susanth, Solly, Vipindas, Rehitha, Ponnoottan, Jabir, Mishriya, Thathu, Adarsh, Bijesh, Ashwini for making my Goan diaries complete.

I also express my deepest gratitude to my lovely parents Mr. N. V. Thomas and Mrs. Usha Thomas and my big little brother Felix for their continual support and prayers throughout my PhD life. I express my wholehearted thanks to Mr. Harikrishnan. G for being my pillar in all these years of my research and for encouraging me to overcome all my failures. I also extend my thanks to my best friend Ammu and my cousins for constantly keeping in touch with me throughout my PhD life.

Above all I bow my head before the God Almighty, who blessed me with health and confidence that made me stand in good for the successful completion of my Research work.

Femi Anna Thomas

Dedicated to

My family and my Guide Late Dr. K. P. Krishnan

Contents

Abstract

The Arctic forms an important part of the cryosphere and is considered a 'global hotspot' highly sensitive to climate change. The Svalbard archipelago situated in the High-Arctic is a highly vulnerable ecosystem known for the presence of rapidly retreating glaciers and associated estuarine fjords. These systems are studied extensively to understand their ecosystem dynamics in response to climate warming. The accelerated retreat of both tidal and land-terminating glaciers is known to influence the biogeochemical cycling of nutrients as well as the microbial community structure in downstream fjord systems. The degree to which downslope transport of microbes from the glacier systems along a hydrological continuum contributes to varying patterns of microbial diversity in the downstream fjord ecosystems and their ecological dynamics is a lacuna in the Norwegian High Arctic region.

Microorganisms play a crucial role in nutrient remineralization and biogeochemical cycling and constitute the basis of trophic networks, thereby influencing the Arctic ecosystem dynamics. With Arctic warming and reports on the long-range transport of pollutants like metals via atmospheric and oceanic circulation along with the localized metal sources in the Arctic, it is essential to understand the microbial community structuring in different Arctic habitats and their role in the fate of contaminants. Considering these aspects in mind, the present thesis titled "**Bacterial diversity and its interaction with metals in glacio-marine environment of Arctic**" uses a combination of cultivation-dependent and cultivation-independent techniques along with metabolic profiling, to understand the bacterial community structure associated with a land terminating glacier-Vestrebroggerbreen glacier, its associated meltwater channels and the downstream system of Kongsfjorden. The study also demonstrates how Arctic bacteria can tolerate metals of ecological significance (Hg, Pb, Cd, Ni, Co, Zn, and Mn), the different metal-bacterial interactions and their genomic mechanisms for tolerance.

Significant variation ($R = 0.873$, $p = 0.001$) in the bacterial community structure between the Vestrebroggerbreen valley glacier, its associated meltwater channels,

and the downstream marine system of Kongsfjorden was observed suggesting the influence of environmental variables and dispersal vectors in determining the patterns of bacterial diversity. Furthermore, the study highlights the importance of different Arctic hotspots to explore the unknown microbiota. The bacterial community from the MWs were found to be true signatures of the glacier ecosystem while the Kongsfjorden bacterial fraction mostly represented heterotrophic marine taxa influenced by warm Atlantic waters and the presence of organic matter. Significant differences were also observed in the carbon substrate utilization profiles between the terrestrial and fjord sediments at both 4 °C and 20 °C incubations ($p < 0.005$) implying the adaptive responses of bacterial members to utilize the available substrates in the varying natural environment. Utilization of carbon substrates such as N-acetyl-D-glucosamine, D-mannitol, and Tween-80 by the sediment communities and individual bacterial isolates from the terrestrial and fjord systems, irrespective of their temperature incubations suggested the affinity of bacteria for such substrates as energy sources and their survival strategies in cold environments. The significant influence of temperature on the growth response of terrestrial and fjord isolates towards different carbon substrates was indicated in our study, with phylogeny found to be a less important structuring component of bacterial metabolic potential.

From the metal tolerant bacterial diversity analyses for the glacio-marine samples, the highest toxicity of Hg for bacterial cells was indicated, with decreasing order of toxicity as Hg > Zn > Co > Cd > Pb > Mn > Ni. Kongsfjorden samples yielded the highest total retrievable bacterial counts in different metal amendments as compared to the glacier snout and foreland samples, suggesting the presence of more resistant bacterial members in the fjord as compared to the glacier samples. This very well corroborates with the higher background trace metal concentrations observed for the fjord samples in comparison to the glacier and foreland samples. The dominant metal tolerant bacterial fraction belonged to the class γ-proteobacteria (45% of the total), α-proteobacteria (19.8%), and Actinobacteria (15.8%). It was observed that the glacier and the fjord system have different metal tolerant bacterial species exhibiting varying levels of tolerance towards the metals tested. Selected bacteria belonging to the genus *Psychrobacter, Planococcus,* and *Halomonas* exhibited multi-metal tolerance and effectively removed metals in

enrichment studies. Pb removal efficiency was significantly higher for all three bacterial isolates (53.75% for HM11, 48.57% for HM12, and 56.66% for HM116 cultures incubated for 14 days) in comparison to the metals Cd, Ni, Co, Zn, and Mn ($p<0.05$). First of its kind, the work suggests the potential of Arctic bacteria for bioremediation applications in Polar Regions with well-developed metal resistant, antibiotic/multi-drug resistant genes and potential efflux pump mechanisms in their genomes.

The observations from this study have been published in reputed international journals such as Science of the Total Environment, Antonie van Leeuwenhoek and International Journal of Systematic and Evolutionary Microbiology.

Keywords: Arctic, Glacio-marine, Bacterial diversity, Metabolic profile, Metals, Genomic mechanisms.

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Abbreviations used in the thesis

Abbreviations	Description
SAT	Surface air temperatures
Cu	Copper
Zn	Zinc
Mn	Manganese
Co	Cobalt
Mo	Molybdenum
Hg	Mercury
Cd	Cadmium
Pb	Lead
AW	Atlantic Water
α-Proteobacteria	Alpha-Proteobacteria
β-Proteobacteria	Beta-Proteobacteria
γ-Proteobacteria	Gamma-Proteobacteria
AMAP	Arctic Monitoring and Assessment Programme
RND	Resistance, nodulation, cell division
CDF	Cation diffusion facilitator
VB	Vestrebroggerbreen
MW	Meltwater
Cl^-	Chloride
SO_4^{2-}	Sulphate
NO_3^-	Nitrate
NO_2^-	Nitrite
SiO_4^{2-}	Silicate
PO_4^{3-}	Phosphate
TDS	Total dissolved solids
TOC	Total organic carbon
IC	Ion chromatography
ICP-MS	Inductively coupled plasma mass spectrometry
DAPI	4, 6-diamidino-2- phenylindole
PBS	Phosphate buffered saline

ABM	Antarctic bacterial medium
TSA	Tryptone soy agar
LB	Luria Bertani medium
AIA	Actinomycete isolation agar
ZMA	Zobell marine agar
ASW	Artificial seawater
Rep-PCR	Repetitive element sequence-based Polymerase Chain Reaction
SRA	Sequence read archive
OTU	Operational taxonomic unit
ANOSIM	Analysis of similarities
SIMPER	Similarity percentage
ANOVA	Analysis of variance
db-RDA	distance-based Redundancy analysis
TMC	Total microbial count
RHBC	Retrievable heterotrophic bacterial count
CFU	Colony forming unit
NGS	Next generation sequencing
CLPP	Community-level physiological profiling
NCPOR	National Centre for Polar and Ocean Research
AWCD	Average well color development
OD	Optical density
MM	Minimal media
CCA	Canonical correspondence analysis
PCA	Principal component analysis
IC	Inorganic carbon
GlcNAc	N-acetyl-D-glucosamine
NJ	Neighbor-Joining
nMDS	Nonmetric multidimensional analysis
BV	Baseline value
MIC	Minimum inhibitory concentration
EPS	Exopolysaccharide
MBM	Modified basal medium

CMC	Carboxy methyl cellulose
TBA	Tributyrin Agar
ICP-OES	Inductively coupled plasma-optical emission spectrometry
ARG	Antibiotic resistance gene
CDS	CoDing Sequences
SCG	Single-copy marker gene
HMM	Hidden Markov Model
ANI	Average Nucleotide index

Chapter 1.

Brief Introduction

Chapter. 1.
Brief Introduction

A major portion of the Earth's biosphere is spread across the Polar Regions and the cold oceans (Priscu and Christner, 2004). The Arctic is an important part of the cryosphere comprising of heterogeneous habitats created by gradients of geomorphology, latitude, proximity to coasts, and oceanic currents (Miteva, 2008). Over the last few decades, it has been reported that the Arctic is warming at a rate that is three times the global average rise in temperature and this has resulted in enhanced glacier retreat, sea-ice decline, permafrost thawing, decrease in the extent and duration of snow cover and increase in the primary productivity in the region (Semenov, 2012). The Svalbard archipelago in the Arctic hosts a set of unique ecosystems which include the tidal glaciers, valley glaciers, glacial fjords, tundra, lakes, rivers, and meltwater streams (Reddy et al., 2009). Many of these systems are interconnected and are highly influenced by the climate change implications through alterations in their physical, biogeochemical, and biological environments (Vincent et al., 2011). The tidal and land terminating glaciers, which account for about 57% of the total Svalbard landmass (Nuth et al., 2013), play a critical role in the downstream ecosystem dynamics. The fjord systems where the glaciers are emptying into, form one of the major downstream aquatic ecosystems to the glaciers and act as sentinel systems to understand the climate warming implications. This is best explained by the West Spitsbergen fjords in Svalbard which receive Atlantic waters through the fjord mouth and glacial influx in the inner parts of the fjord (Svendsen et al., 2002). Glacier materials are altered physically and chemically before they are released into the fjords either beneath the glaciers or in the case of land terminating glaciers during the transport from the glacier terminus to the fjord (Hodson et al., 2008). Such glacio-marine ecosystems despite their extreme conditions such as low temperatures, seasonal fluctuations in ice cover, the extreme seasonal shift in solar radiations, high UV exposure in summer, etc., are mainly dominated by diverse microbial communities. These microbes are the key

players in the biogeochemical cycling and nutrient remineralization processes, thereby supporting the higher trophic levels in the food chain (Cavicchioli, 2015). In the light of climate warming, it would be imperative to understand the effect of climatic perturbations on the microbial community structure and functions, since they form ideal sentinels of climate change (Dinasquet et al., 2018). In this regard, the marine and freshwater environments in Svalbard, Arctic offers numerous distinct habitats to study the microbial community structure and their varying functional profiles.

The microbial community structure present within each of the different environmental compartments like snow, ice, meltwaters, marine fjord waters, and sediments are distinct and their functions reflect the ecosystem conditions. The degree to which downslope transport of microbes from the glacier systems along a hydrological continuum contributes to varying patterns of microbial diversity in the downstream ecosystems and their ecological dynamics is a lacuna in the Norwegian High Arctic region (Thomas et al., 2020).

Another important aspect that is of concern is the increased release of contaminants into the Arctic (Hauptmann et al., 2017). There are numerous studies suggesting the long-range transport of pollutants especially metals, via atmospheric and oceanic circulation. These could have strong implications on the different Arctic ecosystems (MacDonald et al., 2000; AMAP, 2005; Bazzano et al., 2014). For instance, elevated concentrations of contaminants like metals can significantly impact the microbial community structure and their functions (Kandeler et al., 2000).

Studies on extreme Arctic environments characterized by a still limited anthropogenic influence in comparison to the global scenario are critical, since they may give insights into specialized microbial members having novel genomic machinery to combat the toxic elements in their system (Romaniuk et al., 2018). Understanding how these metal contaminants get distributed within different Arctic ecosystems, how it is affecting the microbiota of each system, and whether there is a selection of resistant microbial population in response to the metal enrichments or metal types need special emphasis. Our study mainly focuses on understanding the bacterial community structuring and diversity associated with a land terminating glacier - Vestrebroggerbreen located in

Ny-Ålesund, Svalbard, its foreland channels, and the downstream system of Kongsfjorden with special emphasis on the trace metal distribution and the identification of metal tolerant bacterial fraction in the connected systems. An experimental and genomic approach to understanding the metal-bacterial interactions is also undertaken to assess the inherent potentials of Arctic bacteria to combat the increasing metal contaminants in the pristine Arctic. To our best knowledge, this is the first complete record following a multi-habitat approach highlighting the total bacterial community, metal tolerant bacterial fraction, and the metal-bacterial interactions from an interlinked glacio-marine system in the Arctic.

Chapter 2.

Review of Literature

Chapter. 2.

Review of literature

Polar Regions are the areas contained within the Arctic and Antarctic Circles, dominated by extreme cold conditions and the presence of ice, snow, and water (Convey et al., 2008). Polar regions receive significantly lower amount of solar radiation in comparison to the other regions, leading to lower mean temperatures which in turn strongly limits the biota. The Arctic is a frozen ocean surrounded by continental landmasses and open oceans, whereas Antarctica is a frozen continent surrounded solely by oceans. The Arctic is influenced strongly by seasonal atmospheric circulation and riverine influence from surrounding continents while the Antarctic region is thermally isolated from the rest of the planet by the surrounding Southern Ocean and the atmospheric polar vortex. Both regions have a major influence on the global climate (Anisimov et al., 2001).

2.1. An introduction to the Arctic

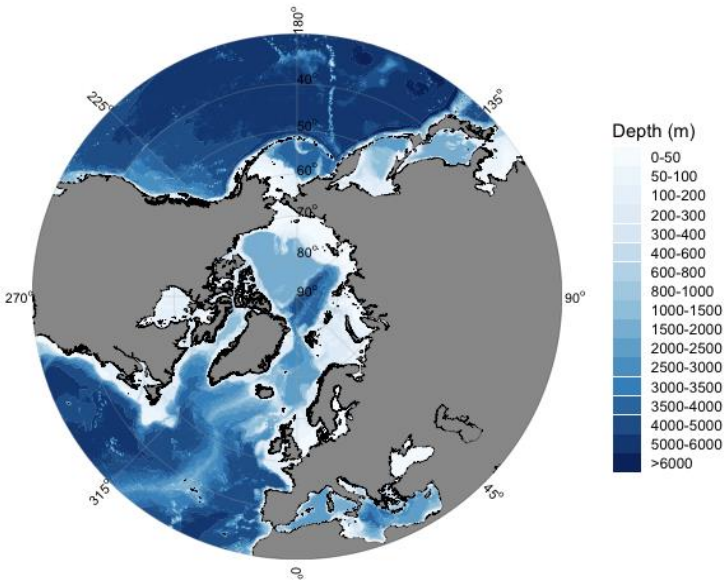

Fig. 2.1. Base map of Pan-Arctic with bathymetry (Vihtakari, 2020). *https://github.com/MikkoVihtakari/PlotSvalbard*

The Arctic is an important part of the cryosphere comprising of terrestrial, freshwater, and marine environments geographically distributed across northern Asia, Europe, and North America. The short growing seasons with low temperatures, snow and ice cover, extreme seasonality along with several distinct habitats such as sea ice, glacial ice, permafrost, tundra wetlands, subglacial soil, periglacial soil, oceanic water, fjords, etc. (Reddy et al., 2009) stands significant in structuring its biodiversity.

2.2. The changing Arctic

The Arctic region is undergoing rapid environmental changes as a function of climate variability (Hop et al., 2002; Makhalanyane et al., 2016). Over the past few decades, seasonal minimal sea ice extent throughout the Arctic has declined by 45,000 km^2/year (Parkinson and Cavalieri, 2008) (**Fig. 2.2**). Surface air temperatures (SAT) which represent one of the strongest indicators of Arctic climate change has shown a strong positive trend towards warming pan-Arctic over the last five decades (Box et al., 2019) (**Fig. 2.3**). The rise in Arctic SAT has resulted in amplification of glacier retreat, decreased mass balance of ice sheets and glaciers, reduction of snow cover, and increased permafrost temperatures (Hansen et al., 2010; Hanna et al., 2021; Biskaborn et al., 2019). Multidecadal SAT warming has also been a strong factor influencing increased trends in terrestrial vegetation productivity and "greening" of the Arctic tundra (Myers-Smith et al., 2020). This, in turn, influences climate change at lower latitudes through changes in river runoff, effects on global thermohaline circulation, impacts on atmospheric circulation, and modulation of atmospheric CO_2 and CH_4 concentrations (Overpeck et al., 1997).

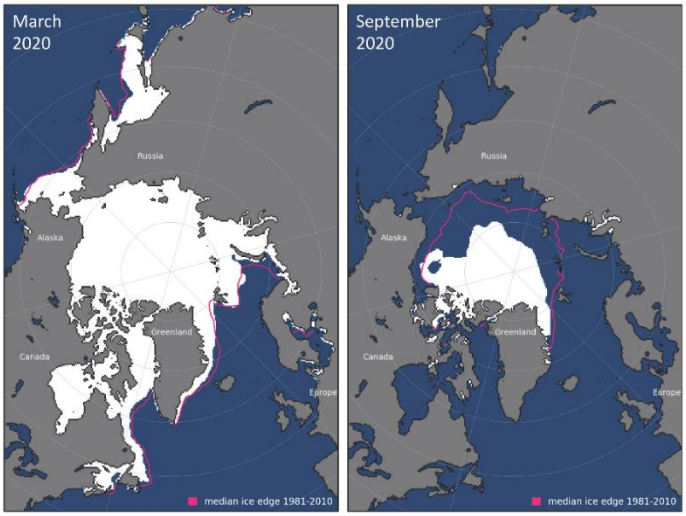

Fig. 2.2. Monthly average sea ice extent map for March 2020 (left) and September 2020 (right), Perovich et al., 2020 (*https://doi.org/10.25923/n170-9h57*)

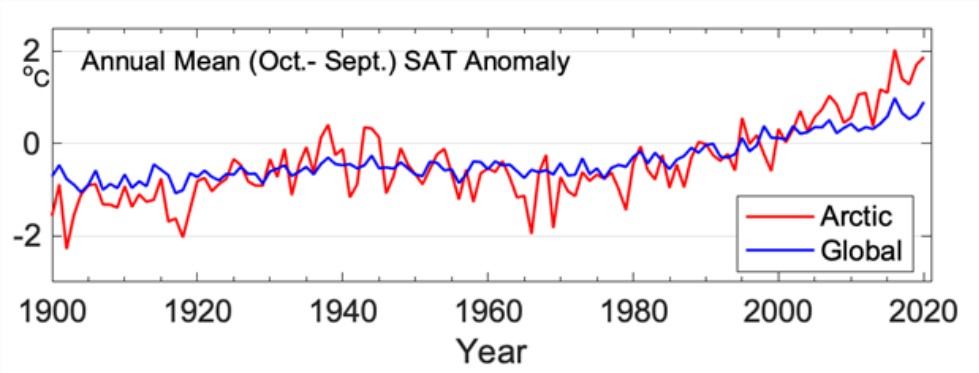

Fig. 2.3. Mean annual surface annual temperature (SAT) anomalies (in °C) for terrestrial weather stations located in the Arctic (60-90° N; red line) and globally (blue line) for the 1900-2020 period, relative to the 1981-2010 means, Ballinger et al., 2020 *(https://doi.org/10.25923/gcw8-2z06).*

Arctic environments are also facing several threats due to anthropogenic activities and are known to receive pollutants from localized as well as sources far outside the Arctic region (de Wit et al., 2022; Townhill et al., 2021; AMAP, 2018; AMAP, 2005; Macdonald et al., 2000). With the increasing pressures from settlements, resource extraction, mining, the opening of numerous commercial shipping routes, and tourism in the Arctic, the Arctic Ocean is predicted to be nearly ice-free in the summer by 2050 (AMAP, 2011). The major contaminants including heavy metals, polychlorinated biphenyls (PCBs), persistent organic pollutants (POPs), and microplastics make their entry into the Arctic via atmospheric deposition and oceanic circulation. The contaminant transport, release, and their distribution within different Arctic compartments are driven by both natural and anthropogenic processes and are highly influenced by climate variability and global climate warming scenarios (AMAP, 2005; Bazzano et al., 2014).

2.3. Metals in the Arctic

Metals occur naturally in the environment in the form of ions, vapors, salts, and minerals. Some metals are essential for living organisms as micronutrients while certain metals can be toxic and pose threat to the biota even in trace amounts. For example, metals such as copper (Cu), zinc (Zn), manganese (Mn), cobalt (Co) etc. are biologically important at low concentrations, but their elevated concentrations and long-term exposures produce detrimental effects on living cells and processes. On the other hand, metals like mercury (Hg), cadmium (Cd), and lead (Pb) are toxic even at very low concentrations (Nies, 1999). Heavy metals can affect the ecosystem's health adversely due to their toxicity and bioaccumulation in different trophic levels in the food chain.

The Arctic region acts as a major receptor of heavy metals produced in other regions of the Northern Hemisphere as well as sources outside the Arctic. Based on existing emission estimates from the Arctic, the main anthropogenic sources of heavy metals to the atmosphere include fossil fuel combustion, waste incineration, and non-ferrous metal production (AMAP, 2005). Coal combustion contributes mercury, manganese,

antimony, selenium, etc. to atmospheric emissions. Combustion of leaded, low-leaded, and 'unleaded' gasoline continues to be the major source of atmospheric lead (Pb) emissions while oil combustion contributes to the addition of nickel. Emissions during non-ferrous metal production account for the biggest source of atmospheric cadmium, copper, and zinc. Also, natural sources such as volcanic releases, soil-derived dust, biogenic sources, and sea salt aerosols emit significant amounts of heavy metals to the atmosphere (AMAP, 2005). These natural sources can influence the freshwater, terrestrial, and marine environment and are difficult to assess than the atmospheric sources. These are suggestive that both anthropogenic activities, as well as the natural processes in the Arctic, can have direct effects on the environment through the release, distribution, and deposition of metals, thereby affecting the biota, ecosystem processes, and changes in precipitation regimes (Blaud et al., 2015).

2.4. An Introduction to Svalbard, Arctic

Svalbard is an archipelago situated in the High Arctic to the north of the European mainland covering a total area of about 61,022 km^2. Situated between 76°N and 80°N, the archipelago is bordered by the Arctic Ocean to the north, the Barents Sea to the south, and the Norwegian Sea to the west (Hald and Korsun, 1997) (**Fig. 2.4**). The Svalbard archipelago is one of the places in the Arctic where rapid changes in landscape forms have occurred due to deglaciation (Kim et al., 2017). Glaciers cover about 57% of the total surface area in Svalbard and many of them are reported to be rapidly retreating over the past 30 years (Nuth et al., 2013). The glacierized area contains a mix of tidal and valley/land-terminating glaciers, ice fields, and ice caps. Most of the glaciers in Svalbard are polythermal exhibiting surge-type behavior with negative surface mass balance reported so far (Nuth et al., 2013).

The Svalbard archipelago is also known for the presence of fjords having a typical glacial morphology. The ecology and biogeochemistry of the Svalbard fjords are strongly influenced by the Arctic amplification and ocean warming scenarios (Jørgensen et al., 2021), hence studied extensively for understanding modifications of the ecosystem structure and functioning (Cottier et al., 2005). The west Spitsbergen

fjords are influenced by relatively warm and saline Atlantic Water (AW), relatively cold and less saline Arctic/Polar water, and glacial meltwater (Cottier et al., 2005; Ślubowska-Woldengen et al., 2007; Nilsen et al., 2008).

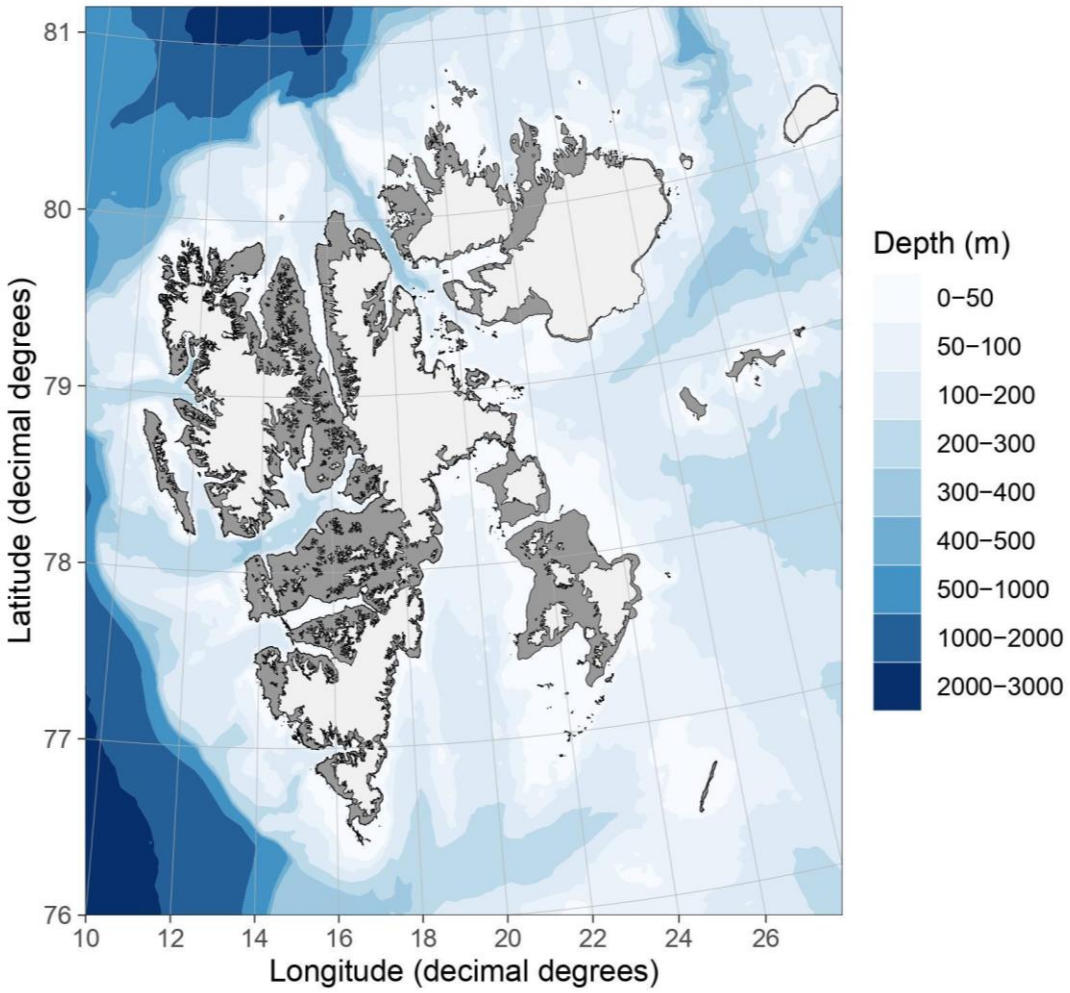

Fig. 2.4. Base map of Svalbard (*https://github.com/MikkoVihtakari/PlotSvalbard*)

2.5. Linking glaciers to the downstream systems

The net effect of glacier discharge on fjord productivity depends on glacier type (marine or land-terminating) and fjord-specific spatiotemporal variability of environmental conditions, such as light, nutrient availability, or fjord-glacier topography (Hopwood et al., 2020). The subglacial discharge plumes from tidal glaciers transport nutrient-rich deep water while the river runoff from land-terminating glaciers contributes marginally to the available macronutrients for fjord productivity (Hopwood et al., 2018).

The land terminating/ valley glaciers impart unique footprints on the downstream ecosystems such as the fjord systems by causing shifts in biogeochemical and sediment fluxes (Hawkings et al., 2016), biodiversity loss (Brown et al., 2007; Jacobsen et al., 2012), and altered food web dynamics (Gutiérrez et al., 2015). The accelerated retreat of glaciers results in terrigenous inputs from various sources, *i.e.* aeolian and guano inputs, glacial flour particle release (Mindl et al., 2007), and the transport of entrapped organic and inorganic compounds (Zarsky et al., 2013). This in turn influences the biogeochemical cycling of nutrients as well as the structuring of microbial communities in the downstream ecosystems. Interactions between ice-covered land, freshwater from retreating glaciers, and marine ecosystems contribute significantly to the Arctic microbial food web (Fellman et al., 2010, Harding et al., 2011).

2.6. Microbiome of Arctic glaciers and fjords

The dominant life forms of Polar environments are microbes which include psychrophilic and psychrotolerant bacteria, archaea, and the dominant photosynthetic eukaryotes primarily diatoms. The highly diverse microbial assemblages associated with the high Arctic glaciers and marine ecosystems play a critical role in nutrient remineralization (Hodson et al., 2008; Skidmore et al., 2005), impact hydrochemistry (Hodson et al., 2002), and are responsible for the metabolism and transformation of environmental contaminants (Barkay and Poulain, 2007). As the key members of the microbial community, bacteria have a significant role in driving the biogeochemical cycling of nutrient, thereby facilitating colonization by subsequent trophic levels. The

different physiological strategies that enable the bacteria to withstand the extreme cold conditions include increased membrane fluidity (Kumar et al., 2002), synthesis of cold-adapted enzymes (Singh et al., 2014; Prasad et al., 2014), and production of cold shock and antifreeze proteins (Cloutier et al., 1992; Gilbert et al. 2005). It is indeed interesting to note bacterial activities even at sub-zero temperatures in sea ice and snow (Junge et al., 2004; Panikov and Sizova, 2007). Bacteria are also able to survive exposure to high levels of solar UV-B (280-314 nm) radiation over the Arctic regions during the summer (Knudsen et al., 2005). Several studies on bacterial diversity in the Svalbard region mainly focused on the identification and characterization of retrievable fraction from snow cover (Amato et al., 2007), cryoconites (Singh et al., 2014), glacier forefields (Reddy et al., 2009), and Kongsfjorden (Prasad et al., 2014; Srinivas et al., 2009; Sinha et al., 2017a, Jain and Krishnan, 2017a). The recent advances in ecological studies using high throughput sequencing approaches for the environmental samples have exceeded the capacity of traditional Sanger sequencing-based approaches by several orders of magnitude, with the possibility of detecting even very rare microbial taxa (Callahan et al., 2019). Hence, many studies were carried out using 16S rRNA gene clone libraries, pyrosequencing, and amplicon sequencing to understand in detail the bacterial community structure associated with different ecosystems in the Arctic.

To highlight a few, Larose et al. (2010) reported the microbial diversity from seasonal snow and meltwater samples from Svalbard using 16SrRNA gene clone libraries and suggested significant differences between the snow and meltwater clone libraries. They have also noted higher community richness and biodiversity indices for meltwater libraries as compared to the snow libraries. The bacterial diversity in the surface soils along the glacier foreland of MidtreLoven glacier was assessed using 454 pyrosequencing by Schuette et al. (2010) and reported a significant positive correlation between changes in phylotype richness and evenness along the chronosequence of the glacier foreland. A similar pyrosequencing approach by Kim et al. (2017) revealed that both deglaciation time and environmental factors (pH and soil temperature) significantly influence the bacterial community structuring in different stages of succession in the foreland regions of Austre Lov´enbreen glacier, Svalbard. Lutz et al.

(2017) evaluated the distinct habitats on glacial surfaces including snow, biofilms, dirty ice, and cryoconite holes for their microbial community composition (of algae, bacteria, and archaea) using 16SrRNA gene amplicon sequencing approach in combination with several indicators of microbial functionality such as fatty acids and pigments and revealed clear differences between the communities in these habitats. Similarly, Kosek et al. (2019) studied an aquatic system of the Revelva catchment (Spitsbergen), based on summer samples collected from the lake, river, and their tributaries using V3-V4 16SrRNA gene amplicon sequencing approach and indicated that the bacterial community structure was characterized by high biodiversity indices and development of multiple survival strategies and metabolic potentials to withstand the oligotrophic conditions in the system.

Some recent high throughput sequencing studies from Svalbard marine systems include Zeng et al. (2013), Sinha et al. (2017b), Jain and Krishnan. (2017b), Jain et al. (2019), Han et al. (2021) from the fjord waters, Zeng et al. (2017), and Fang et al. (2019) from the fjord sediments. Studies from the Kongsfjorden waters suggest the strong and localized influence of glacial meltwater in shaping the bacterial community structure of fjord waters (Sinha et al., 2017b); clear niche differentiation between the particle-associated and free-living bacterial fraction in the fjord waters (Jain et al., 2019); distinct niche patterns in the Kongsfjorden waters as influenced by different water masses and habitat transition between Atlantic and fjord surface waters (Han et al., 2021). Similarly, studies from the Kongsfjorden sediments using 454 pyrosequencing approach by Zeng et al. (2017) reported the dominance of bacterial genera like *Sulfurimonas* and *Sulfurovum* in the fjord sediments, suggesting the increased influx of Atlantic waters into the Kongsfjorden influencing the outer and inner fjord sediment community composition. The V3-V4 16SrRNA gene amplicon sequencing analysis of the Kongsfjorden sediments by Fang et al. (2019) indicated significant differences in the community structure between the inner, central and outer fjord sediments with location depth, temperature, and salinity being the significant environmental factors having a strong correlation with the community.

The bacterial sequences reported from the glacier, foreland and fjord ecosystem of Svalbard mostly fell under the major bacterial phyla- Proteobacteria (α-Proteobacteria, β-Proteobacteria, and γ-Proteobacteria), Actinobacteria, Bacteriodetes (especially Flavobacteria and Sphingobacteria), and Verrucomicrobia (Hell et al., 2013, Larose et al., 2010, Sinha et al., 2017b; Venkatachalam et al., 2021). Although several discrete bacterial studies have been reported from different habitats in the Arctic, an integrated study covering a glacier, its forefields, and the associated downstream fjord ecosystem in Svalbard Arctic has not been undertaken. **A single study encompassing all these cryospheric components is critical to understand how the bacterial community structure differs within these ecosystems and to what extent the glacier ecosystem and its forefields contribute to the fjord bacterial community structuring.**

2.7. Trace metal distribution in Svalbard

The Svalbard archipelago can be considered a pollution reservoir of the Arctic because of its location and prevailing environmental conditions (Ruman et al., 2012). The proximity to the European Continent, makes this archipelago a highly sensitive region, particularly exposed to the pollutants released from the industrial European countries (such as the European part of Russia, Norway, Great Britain) (Kozak et al., 2013; Polkowska et al., 2011). Natural factors like agricultural and forest fires (Cahill et al., 2008; Nawrot et al., 2016), volcanic eruption (Iceland) (Langmann et al., 2012; Karasiński et al., 2014), or glacial runoff (Bazzano et al., 2014; Stachnik et al., 2016) also impact the region. Hunting and industrial activities like mining for resource extraction were active in the Svalbard archipelago throughout historic times. Currently, there are three active coal mining sites in Svalbard (Svea, Longyearbyen, and Barentsburg) along with several abandoned mine facilities on the main island (Spitsbergen). This region is also known to have vast resources exceeding about 6 billion tons of oil equivalents (Dahle et al., 2006). Resource extraction hence accounts for a major local source for pollution in Svalbard (Schmale et al., 2018). The anthropogenic impact is also related to coal and diesel combustion driven from power plants, shipping, as well as tourist and research-related traffic (**Fig. 2.5**).

The heavy metal contamination of the Svalbard fjord sediments has been monitored by Arctic Monitoring and Assessment Programme (AMAP) since the 1990s. Many studies are reporting the trace metal distribution from the different fjord sediments (Lu et al., 2013; Grotti et al., 2013 and 2017; Zaborska et al., 2017; Mohan et al., 2018; Herbert et al., 2020; Choudhary et al., 2020) and suspended particulate matter in the fjord waters (Bazzano et al., 2014) in Svalbard. Most of these studies focussed mainly on the glacial fjord Kongsfjorden located in the West Spitsbergen, Svalbard since it forms an ideal laboratory for studying the impact of climate change and anthropogenic influence on the Arctic ecosystem. This fjord is influenced by the oceanic waters on the outer side and the glacial discharge from the tidal glaciers on the inner side of the fjord (Svendsen et al., 2002). The riverine discharge also brings in glacier flour and sediments from the land terminating glaciers into the fjord. The Kongsfjorden is also influenced locally by anthropogenic activities occurring at Ny-Ålesund, where several research stations have been established by several countries, making the site highly vulnerable to human impacts. There are also reports on the transport of anthropogenic metals from lower latitudes by Atlantic waters in the outer part of the fjord while towards the inner part of the fjord, the trace element sources can be linked to the inputs of inorganic matter from glacial run-off and pyrite precipitation (Lu et al., 2012, Bazzano et al., 2014; Grotti et al., 2017).

Similarly, trace element studies were reported from glacier snow samples (Koziol et al., 2021), cryoconite samples (Łokas et al., 2016), riverine catchment samples (Kozak et al., 2016), and terrestrial soil samples (Hao et al., 2013; Halbach et al., 2017; Marquès et al., 2017; Aslam et al., 2019). Trace metal concentrations in the snow samples from the Hansbreen glacier in Svalbard indicated long-range transported volatile elements, elements with crustal sources, and elements released from sea spray aerosol sources in the snow (Koziol et al., 2021). Łokas et al. (2016) reported higher heavy metal concentrations in the cryoconite samples in comparison to the natural background indicating the ability of cryoconite granules to concentrate airborne metallic contaminants. Likewise, concentrations of metals like Al, Cu, Fe, and Pb in the terrestrial compartments of Svalbard appeared to be influenced by the crustal

material, while concentrations of Hg, Zn, and Cd seemed to be affected by atmospheric deposition (Halbach et al., 2017; Aslam et al., 2019).

The glacier environments act as an important source of trace elements to the downstream systems and finally to the ocean (Herbert et al., 2020; Raiswell et al., 2006; Mitchell et al., 2001). Proglacier channels form the conduit transporting sediments from the land terminating glaciers to the fjord systems. The glacier sediments undergo weathering reactions before their release into the fjord systems forming an important source of bioessential trace elements (Anderson et al., 2000; Wadham et al., 2001; Cooper et al., 2002; Herbert et al., 2020). These trace elements may be sequestered in the fjord sediments or recycled back to the water column depending on local biogeochemical conditions and could be a potential threat to Arctic biota if present in excess amounts. **Hence, a detailed study on the trace element distributions in the glacier, its foreland, and the downstream fjord system is crucial to understand how the concentration of different metals varies within the different Svalbard ecosystems and whether the concentrations are exceeding the natural background levels.**

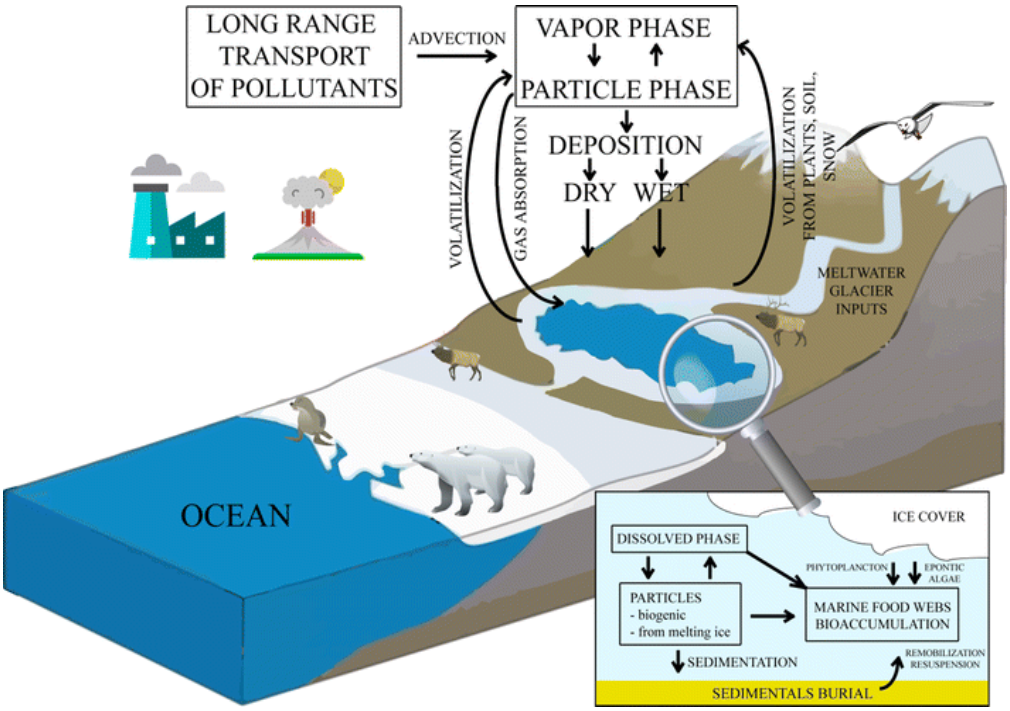

Fig. 2.5. Diagram showing distribution and metal transport pathways in Svalbard, Arctic *(Image Credits: Kozak et al., 2016)*

2.8. Microbes and metals

Microbes interact with metals in natural environments in different ways by altering their physical and chemical state and influencing the biogeochemical cycles of metals. For instance, microbes are known to cause bio weathering of minerals rich in Fe, Ni, Co, Zn, Cd, etc in rocks and soils, Fe solubilization by siderophores, Mn(II) oxidation, and immobilization as Mn(IV) oxides, Mn(IV) reduction, biosorption of Pb, Hg biomethylation, reduction of Hg(II) to Hg(0), oxidation of Hg(0) to Hg(II), Hg volatilization as Hg(0), degradation of organomercurials, etc (Gadd, 2010). Metals in turn are directly and/or indirectly involved in microbial growth, metabolism, and differentiation (Gadd, 1992). Metals interact with microbes in various ways depending on the metal species, organism type, and environment, while the microbes influence the metal speciation, mobility, bioavailability, and toxicity through the different inherent mechanisms and their metabolic activity (Gadd and Griffiths, 1977; Gadd, 2010).

2.9. Metal-bacterial interactions

Metal tolerant bacteria can act as bio indicators of environmental pollution (Trevors, et al., 1985; AMAP, 2011). The response of the bacterial populations to metal contamination in the environment depends on the concentration and bioavailability of metals (Hassen et al., 1998). High concentrations of metals (both essential and non-essential) harm the cells by displacing the enzyme metal ions, competing with structurally related non-metals in cell reactions, and also blocking functional groups in the cell biomolecules (Hetzer et al., 2006). A study by Wang et al. (2010) suggests that environmental stress caused by heavy metals decreases the diversity and activity of bacterial populations leading to a reduction of the total microbial biomass and shift in microbial community structure. Metals are also known to affect bacterial growth, morphology, and biochemical activity (Sandaa et al., 2001; Tsai et al., 2005). For example, Zinc, a micronutrient at elevated concentrations can induce DNA damage, reduce protein and ATP content, interact with enzyme active sites, alter the membrane, and lead to cell death (Vega-López et al., 2007). Metals such as Hg^{2+} and Cd^{2+} tend to bind to SH groups of proteins and inactivate their enzyme functions while other heavy-metal cations may interact with physiological ions i.e. Cd^{2+} interacts with Ca^{2+}, Ni^{2+} and Co^{2+} with Fe^{2+}, Zn^{2+} with Mg^{2+}, inhibiting the functioning of the respective physiological cation (Nies and Silver, 1995). The combined effect of different metals is also known to have higher adverse effects on microbial biomass/activity and diversity than the effects by single metals at high concentrations (Renella et al., 2005). Baltar et al. (2018) also reported largest loss in bacterial abundance at the genus level in all experiments with trace metal mix additions.

The natural exposure of bacteria to bioavailable metals (both essential and toxic) has occurred over billions of years (Barkay et al., 2010), and this exposure has likely been the driver for the evolution of the microbial ability to control cellular levels of these toxic metal ions. Various microorganisms show different responses to heavy metal ions that confer them with a range of metal tolerance (Valls and de Lorenzo, 2002). Bacteria exhibit metal tolerance through biological, physical, or chemical mechanisms that include precipitation, complexation, adsorption, transport, product excretion,

production of pigments, exopolysaccharides, enzymes, and specific metal-binding proteins (Gadd, 1992; Hetzer et al., 2006).

The main mechanisms of metal resistance in bacteria include:

- **Extracellular barrier**
- **Active efflux**
- **Extracellular and Intracellular sequestration and**
- **Reduction of the heavy metal ions to a less toxic state**

(Nies, 1999; Ianieva, 2009)

The cell wall, plasma membrane, or capsule could prevent metal ions from entering the cell, thereby acting as the barrier (Ianieva, 2009). The largest group of metal resistance systems of bacteria is represented by active transport or efflux. Efflux systems consists of proteins belonging to three families: RND (resistance, nodulation, cell division), CDF (cation diffusion facilitator), and P-type ATPases. P-type ATPases and CDF proteins of gram-negative bacteria transport substrates through the plasma membrane into the periplasm. P-type ATPases generally transfer metal ions with high affinity for sulfhydryl groups (Cu+/Ag+, Zn2+/Cd2+/Pb2+) while CDF-proteins specifically interact with ions of divalent metals (Zn2+, Co2+, Ni2+, Cd2+ and Fe2+). Transport complexes formed by RND-proteins transport cations from the periplasm across the plasma membrane (Nies, 2003). Intracellular sequestration in bacteria is enabled by the presence of low molecular weight proteins called metallothioneins while extracellular sequestration is enabled by the cellular components in the periplasm or by metal precipitation as insoluble complexes (Ianieva, 2009). The enzymatic reduction of metal ions results in less toxic metal forms as seen in mercury (Møller et al., 2013) (**Fig. 2.6**). These different tolerance mechanisms are generally plasmid-driven, which greatly contributes to dispersion from cell to cell (Valls and de Lorenzo, 2002) while bacterial

chromosome resistance was also reported in some bacterial species (Abou-Shanab et al., 2007).

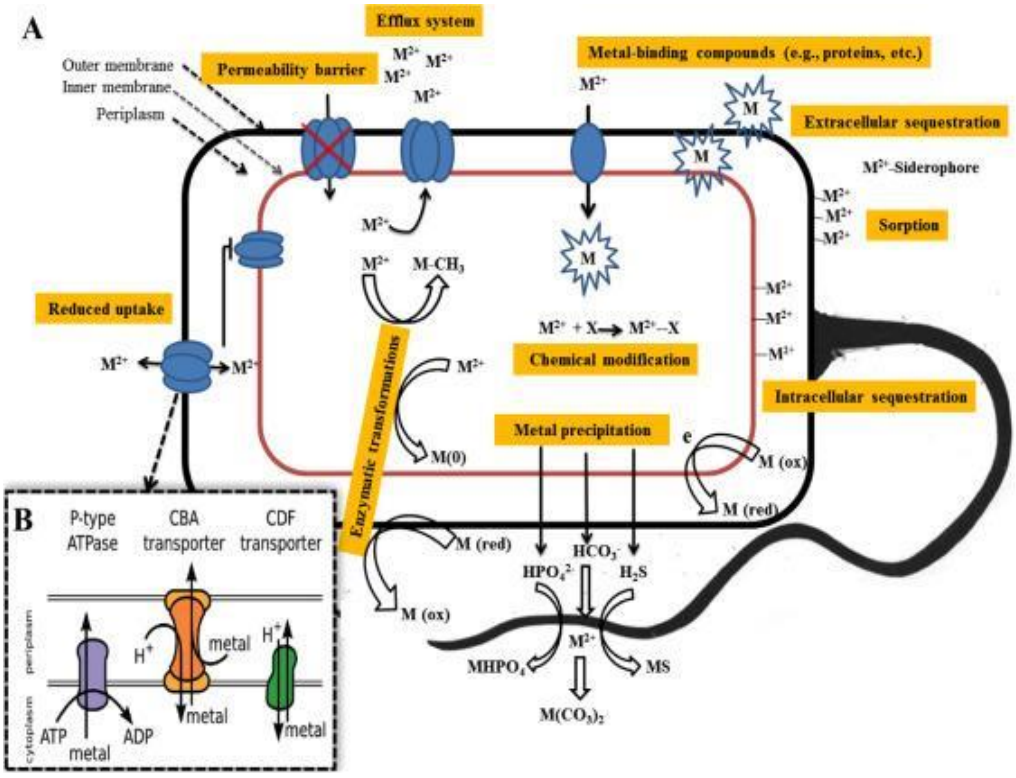

Fig. 2.6. Schematic representation of (A) the different metal tolerance mechanisms by the bacterial cell; M stands for metal cation. The exterior blue barrier represents some external permeability barrier (e.g., biofilm). (B) Main transporter families involved in heavy metal resistance- P-type ATPases pump their substrates from cytoplasm to periplasm using energy provided by ATP hydrolysis. CBA transporters are three-component complexes that span the whole cell wall of Gram-negative bacteria and expel ions from cytoplasm and periplasm to outside using a chemiosmotic gradient. CDF transporters are driven by a proton motive force and they export ions from cytoplasm to periplasm. Image credits: (*Image credits: Etesami, 2018)*

2.10. Metal tolerance in Arctic bacteria

To date, there are only a few studies on metal-resistant bacterial community and their potentials from the Arctic. The potential of mercury tolerant bacteria *Pseudarthrobacter oxydans* and *Pseudomonas frederiksbergensis* isolated from the tundra ecosystem in Svalbard to immobilize mercury from the system was reported by Balan et al. (2018). Møller et al. (2011) and (2014) suggested that mercury-resistant bacteria in the snow could lower the bio-available mercury being incorporated into the Arctic food chains by reducing Hg(II) to Hg(0) and horizontal transfer of merA gene is a likely mechanism to tolerate changing mercury concentrations for High Arctic microbial communities. Larose et al. (2013) has reported a strong correlation between mercury concentrations in snow and the mercury resistance gene copy numbers of snow microbiota. Similarly, the strong influence of local metal inputs into the sediments of the Pasvik area in Svalbard and a positive relationship between the development of bacterial metal resistance and metal contamination in the area was confirmed by Caputo et al. (2019). According to their study, bacterial metal resistance was in the order $Ni^{2+}>Pb^{2+}>Co^{2+}>Zn^{2+}>Cu^{2+}>Cd^{2+}>Hg^{2+}$, with the retrieval of multi-resistant isolates belonging to the genera *Stenotrophomonas*, *Arthrobacter*, and *Serratia.* A direct influence of metal pollutants on the bacterial community distribution in the Pasvik river area was reported by Rappazzo et al. (2019). Characterization of heavy metal resistance in gram-negative bacteria from Kongsfjorden Arctic suggested the level of toxicity of heavy metals in the order of Hg > Cd > Cu > Zn > Pb and a positive correlation between the antibiotic and metal resistance mechanisms for all the isolates tested.

These results are indicative that the cryospheric microbial communities interact with anthropogenic contaminants and might be able to remove a fraction of the contaminants deposited to the environment before they are released to the downstream ecosystems (Stibal et al., 2012). However, detailed studies on the metal tolerant bacterial fraction in the different connected terrestrial, freshwater, and marine environments in the Arctic, their interactions with different metals, and their genomic mechanisms underlying metal tolerance are lacking.

Hence a detailed study was carried out with the following major objectives:

- **To understand the bacterial diversity of glacio-marine environment of Arctic through culture-dependent and culture-independent approach**
- **To assess the diversity of metal tolerant bacteria from the glacio-marine ecosystem of the Arctic**
- **To understand the metal-bacterial interactions so as to elucidate the role of bacteria in modulating the impact of metals in glacio-marine environment**

2.11. Study location

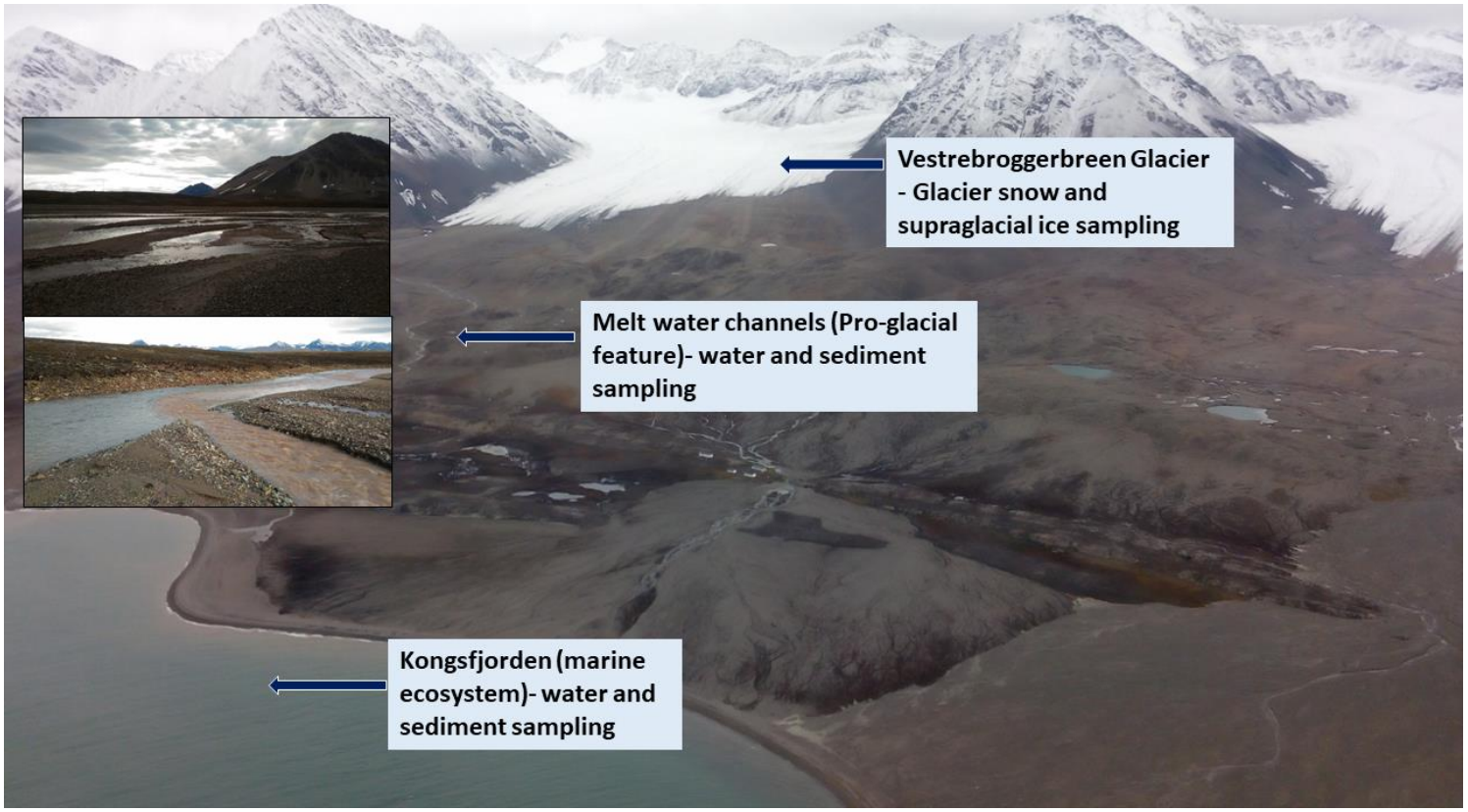

Fig. 2.7. An aerial view of the study location covering the land terminating glacier Vestrebroggerbreen (VB), its meltwater (MW) channels emptying into the Kongsfjorden system.

Chapter 3.

Bacterial community structure of a glacio-marine system in the Arctic (Ny-Ålesund, Svalbard)

Chapter. 3.

Bacterial community structure of a glacio-marine system in the Arctic (Ny-Ålesund, Svalbard)

3.1. Introduction

Glaciers harbor highly diverse microbial assemblages within their different habitats such as snow, surface ice, cryoconite holes, englacial systems, and the interface between ice and overridden rock/soil (Anesio et al., 2017). These microbes might have probably dispersed from close and distant locations with dispersal vectors such as terrestrial dust or aerosols (Margesin and Miteva, 2011) and have the capacity to thrive wherever there is enough liquid water to sustain their activity. During summer, when there is a ready supply of meltwater across much of the glacial cryosphere, microorganisms soon become active in cryoconite holes, supraglacial streams, moraines, and snowpacks. Microbial communities are majorly responsible for changes in the biogeochemistry of runoff that have been identified following the emergence of ice and snowmelt from the glacier surface as well as the glacial bed (Hodson et al., 2008) The Meltwater (MW) channels form connecting link between the glaciers and the downstream habitats like the fjords, by conducting nutrients, sediments, organic, and inorganic debris along with the microbial load (Hop et al., 2002). Although acting as a conduit, these MW channels might create a selection pressure on the associated microbiota due to their dynamic variabilities in the stream discharge volume, sediment, and nutrient flux, enabling the survival of a rather diverse but unstable community (Hotaling et al., 2017; Crump et al., 2007). Conversely, the glacier and fjord systems are known to support more stable cold-tolerant microbial communities which in the case of the glacier are mostly freshwater related and in the case of the fjord are mostly marine-related (Thomas et al., 2020). This suggests distinct patterns of microbial diversity in each of these environmental compartments.

Larose et al. (2010) based on the 16SrRNA gene clone library construction from snow and meltwater samples from Svalbard Arctic have suggested significant differences between the snow and meltwater clone libraries, and highlighted the presence of a common core group of the microbial community within different habitats in the cryosphere. The dominance of class β-proteobacteria in the Arctic glacier snowpack samples was reported by Hell et al. (2013). An amplicon pyrosequencing study by Ntougias et al. (2016) revealed that >70% of the OTUs were shared in common among the stream and lake surface water samples from Fuglebekken and Revvatnet basins of southern Svalbard. The study has also indicated the role of environmental factors like As, Pb, and Sb in influencing the bacterial profiles wherein the communities mainly consisted of freshwater and marine representatives involved in detritus mineralization. Similarly, studies on the Kongsfjorden waters by Sinha et al. (2017b) indicated significant variation between the outer and inner zone bacterial community in the fjord, with the strong influence of glacial meltwater in shaping the bacterial community structure. The prevalence of complex polysaccharide degrading bacterial isolates in the Kongsfjorden waters having phenotypic heterogeneity for substrate degradation was indicated by Jain et al. (2017a) in their culture-based approach.

Likewise, there are numerous reports on microbial diversity, physiology, and functions associated with supraglacial cryoconite sediments (Edwards et al., 2011; Lutz et al., 2017; Poniecka et al., 2020), subglacial sediments (Skidmore et al., 2005; Stibal et al., 2012; Sułowicz et al., 2020) and glacier forefield soils (Kim et al., 2017; Nash et al., 2018; Malard et al., 2019), but studies on glacier snout and forefield associated sediments are limited to a single culture-dependent study from the sediments of a meltwater stream from Midtre-Lovénbreen glacier (Reddy et al., 2009). They have reported the cold-active enzyme production and pigmentation properties of pro-glacier sediment bacteria and indicated the presence of Proteobacteria, Cytophaga-Flavobacterium-Bacteroidetes, and high G+C Gram-positive bacteria as common inhabitants of these habitats. Similarly, many studies are highlighting the microbial community structure (Zeng et al., 2017; Conte et al., 2018; Fang et al., 2019) and functions (Teske et al., 2011; Buongiorno et al., 2019, Jabir et al., 2021) associated

with Svalbard fjord sediments. The increased influx of Atlantic waters into the Kongsfjorden system influencing the sediment community composition between the outer to the inner fjord has been reported by Zeng et al. (2017). Teske et al. (2011) reported higher rates of hydrolytic activities for Kongsfjorden sediment bacterial communities to hydrolyze polysaccharides and algal extracts as compared to the seawater communities. The presence of some microbial key populations (Bacteroidetes) having specific enzymatic capabilities, for example, to readily hydrolyze substrates which were found to be recalcitrant in surface and bottom waters was also noted. Their study further indicated the need to test the linkages between enzymatic potential and phylogenetic identity using cultured isolates. Such studies are indicating that the bacterial communities associated with the Arctic glacio-marine sediments tend to exhibit distinct metabolic properties driven by the substrate availability and the various environmental conditions of each system.

Even though, the aforementioned studies give us an account of the bacterial community composition associated with different Arctic habitats, a single study integrating all these different environmental compartments in the Svalbard Arctic using both culture-dependent and culture-independent approaches has not been undertaken to date. Therefore, in the present study, we focused on a multi-habitat approach integrating the land terminating glacier Vestrebroggerbreen to its pro-glacial networks and the associated downstream marine Kongsfjorden ecosystem to understand the spatial distribution of bacterial community in these interconnected systems, their associations with the various environmental parameters and their metabolic potentials. This chapter comprises of two main themes:

(a) Understanding the bacterial community structure associated with glacier snow and ice, foreland meltwaters, and the downstream fjord waters

(b) Understanding the bacterial diversity and their metabolic potentials associated with the glacio-marine sedimentary environments of Arctic

3(a). Understanding the bacterial community structure associated with glacier snow and ice, foreland meltwaters, and the downstream fjord waters

3(a). 1. Introduction

There could be a gradual decrease in the bacterial diversity on reaching the fjord due to the loss of much freshwater-associated taxa introduced from upstream environments combined with the increase in the relative abundance of a few specific taxa that are best adapted for the marine ecosystem (Thomas et al., 2020). Therefore, this study was undertaken to:

(a) to compare the bacterial community structures in Vestrebroggerbreen (VB) glacier snow and ice, associated MW channels (Bayelva river), and Kongsfjorden ecosystem through culture-dependent and culture-independent high throughput amplicon sequencing approach, and

(b) to elucidate the role of environmental factors such as temperature, major anions (Chloride (Cl^-) and Sulphate (SO_4^{2-})), nutrients (Nitrate (NO_3^-), Nitrite (NO_2^-), Silicate (SiO_4^{2-}), and Phosphate (PO_4^{3-})) and trace metals (Mn, Fe, Ni, Co, Cu, and Zn) in structuring the bacterial community in each of these sampling sites.

This work for the first time provides information on the similarities and differences that exist in the bacterial diversity pattern along the entire transect of this glacio-marine system in Svalbard, Arctic as controlled by dispersal vectors and associated environmental factors (Thomas et al., 2020).

3(a). 2. Materials and Methods

3(a). 2.1. Study Area

The study area is located on the west coast of Spitsbergen, in the Svalbard archipelago (**Fig. 3.1a and 3.1b**). Vestrebroggerbreen (VB) glacier is a 4.7 km^2 long polythermal valley glacier in Ny-Ålesund situated between 78°53'26.9" N - 78°55'14.5" N and

11°38'50.3" E - 11°47'23.3" E (**Fig. 3.1a**). The total glacierized area is over 17 km^2. The lower foothills area of the VB glacier is drained into the Kongsfjorden by seasonal short-length channels. Bayelva river is the principal meltwater channel (~ 4 km long) draining the Brøggerbreen valley glacier into Kongsfjorden (**Fig. 3.1a**). Mostly the river flow is clear in the beginning (early to mid-June) and subsequently becomes turbid and remains so until autumn (September/October) (Zhu et al., 2016). Kongsfjorden is a relatively small fjord (26 km long and 4 - 10 km wide), situated between 78°04′ N - 79°05′ N and 11°03′ E - 13°03′ E (**Fig. 3.1b**). Kongsfjorden is influenced by both Atlantic and Arctic water masses and the inputs from large tidal glaciers create steep environmental gradients in sedimentation and salinity along the length of the fjord. Because of its open connection to Fram Strait, it is directly influenced by variable climate signals in the West Spitsbergen Current (Hop et al., 2006; Walczowski and Piechura, 2011). Advection of transformed Atlantic water into the fjord is important for its seasonal hydrography as well as its biological communities (Cottier et al., 2005; Hop et al., 2006; Willis et al., 2006). The outer fjord is influenced by the oceanographic conditions and the inner fjord is influenced by large tidal glaciers. The inner part of the fjord is influenced strongly by glacial runoff from 5 large tidal glaciers: Kongsvegen, Kronebreen, Kongsbreen, Conwaybreen, and Blomstrandbreen and these glaciers annually supply ~ 0.33 Km^3 of freshwater to the fjord (Svendsen et al., 2002; Beszczynska-Moller et al., 1997).

3(a). 2.2. Sampling strategy

The samples were collected from different geographical transects of the Vestrebroggerbreen (VB) glacier and Kongsfjorden during the Indian Arctic expedition (August-September, 2017). Snow samples were collected from the accumulation and ablation zones of the VB glacier (VBSS1 and VBSS2) while ice sample was collected from the glacier snout region (VBIce). Meltwater samples (MW1-MW6) were collected from the associated pro-glacial channels and Bayelva River and seawater samples from the Kongsfjorden (KNS1, 9, 10, and KNBR) (**Fig. 3.1, 3.2, and 3.3**).

Snow and ice samples were thawed at 4 °C and filtered (~2 L) through 0.2µm GTTP filter papers (Merck Millipore, Germany) and were stored at -80 °C until nucleic acid extraction. Similarly, MW samples (~ 2 L) were also filtered and stored as per the above-mentioned protocol. Fjord water samples (~ 2 L) were collected from the depth of chlorophyll maxima (10 - 50 m) using Niskin bottles and processed for bacterial community analysis as detailed above. Adequate care was taken to aseptically sample and perform various downstream analyses.

On-site measurement of temperature, Total dissolved solids (TDS) and dissolved oxygen in the MWs were carried out using a Waterproof Portable meter (Cyberscan series 600, Eutech Instruments, Thermofisher Scientific, USA). *In-situ* measurement of temperature, salinity and dissolved oxygen in Kongsfjorden was carried out using the CTD profiler (SBE 19 plus V2, USA) deployed from *MS Teisten* (https://nyalesundresearch.no/infrastructures/ms-teisten/).

For the analysis of nutrients and ions, samples were stored (100ml) in a frozen state in polypropylene bottles. For the analysis of total organic carbon (TOC), samples were collected (25mL) in acid-washed (using 0.5% ultrapur HNO_3, Merck Millipore, Germany) and combusted (450 °C, for 4 hours) glass vials and stored at -20 °C. For trace element analysis, samples were acidified with suprapur HNO_3 (Merck Millipore, Germany) and stored until further processing. All the samples were transported in the frozen state to the home laboratory at NCPOR for analysis.

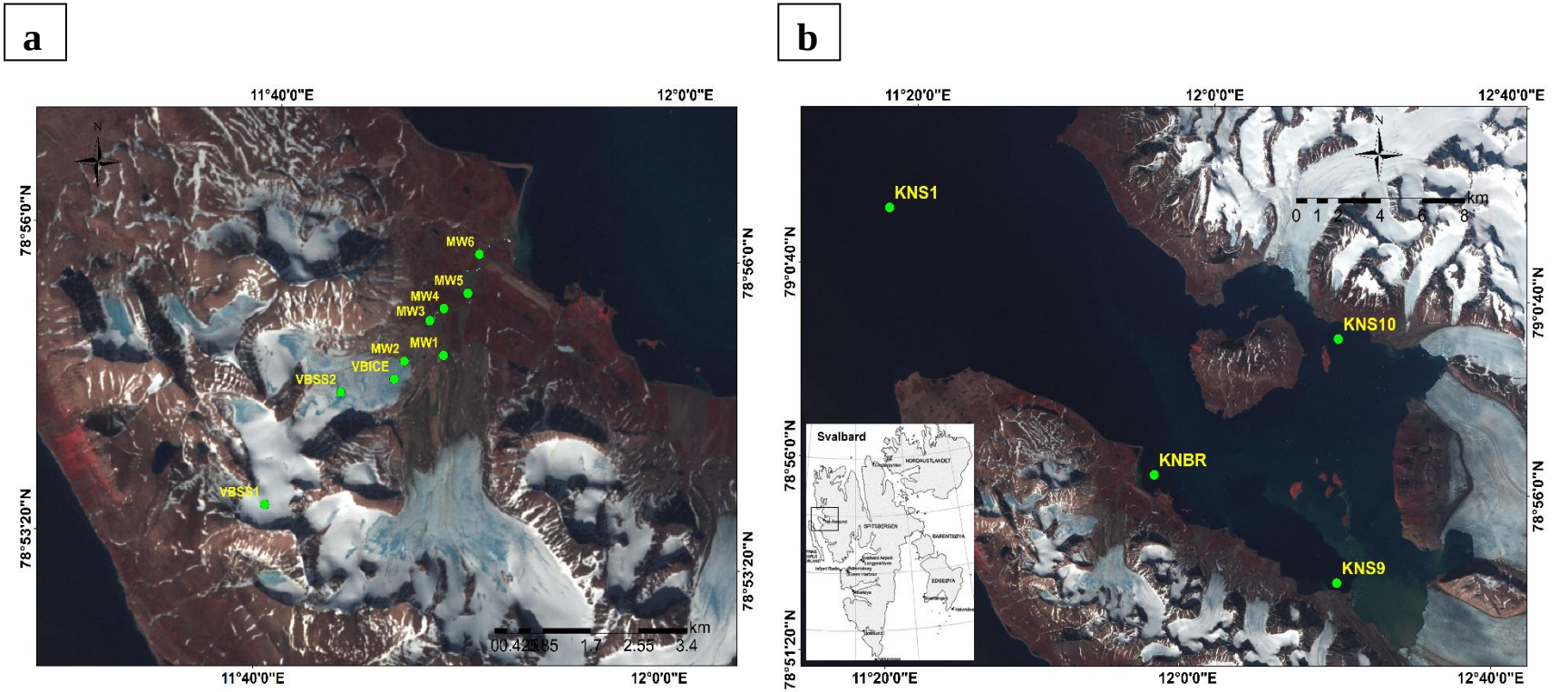

Fig. 3.1(a). Map of Vestrebroggerbreen (VB) glacier and associated Meltwater channels. Snow samples were collected from the accumulation (VBSS1) and ablation (VBSS2) zones of the VB glacier. Ice samples were collected from the VB snout region (VB Ice). Meltwater samples were collected from points starting from the glacier terminus towards the Kongsfjorden and along the Bayelva River (MW1 to MW6). **(b).** Map of Kongsfjorden. Samples from Kongsfjorden were collected from two stations located at inner fjord (KNS9 & KNS10), an outer fjord (KNS1), and from the opening of the Bayelva River into the fjord (KNBR). Map of Svalbard indicating the study area is given in the inset. (https://earthexplorer.usgs.gov/)

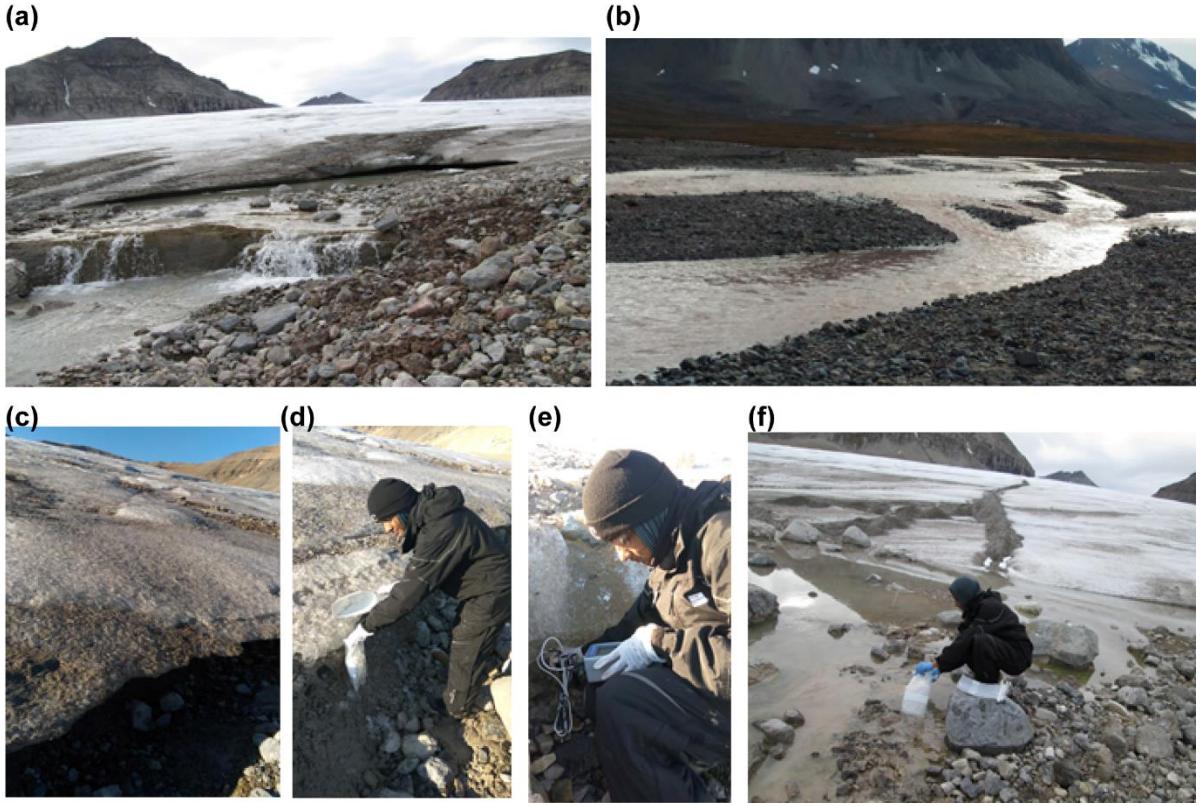

Fig. 3.2. (a) Vestrebroggerbreen (VB) glacier snout region, **(b)** Meltwater channels originating from the Broggerbreen glacier, **(c)** VB snout ice, **(d)** VB snout ice sample collection, **(e)** on-site measurement of meltwater temperature, dissolved oxygen, and total dissolved solids using Waterproof Portable meter, **(f)** meltwater sample collection

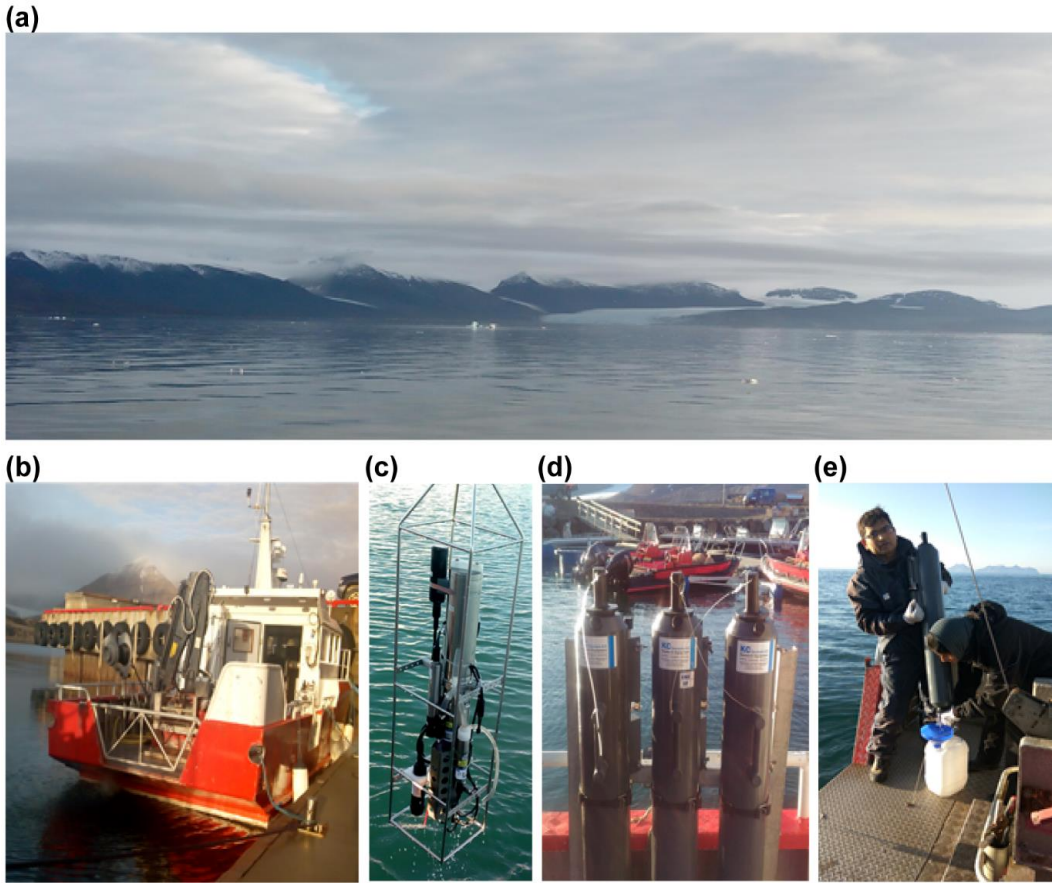

Fig. 3.3. (a) Kongsfjorden, **(b)** *MS Teisten*, the research vessel used for fjord sampling, **(c)** CTD operating from the vessel, **(d)** Niskin water samplers used for water sample collection, **(e)** Water sampling from the research vessel *MS Teisten.*

3(a). 2.3. Chemical analysis

The dissolved nutrients (NO_3^-, NO_2^-, SiO_4^{2-} and PO_4^{3-}) were measured using Seal AA3 analytical auto-analyzer (Wisconsin, USA) following standard colorimetric methods (Grasshoff et al., 1983) with an accuracy of ±1% standard deviation with an R^2 value of 0.999. Total organic carbon analysis was carried out by the high-temperature combustion method (Hansell and Peltzer, 1998) using TOC-TN Analyzer (Shimadzu TOC-L_{CPH}, Columbia, USA). The instrument was calibrated using reagent grade potassium hydrogen phthalate as the organic carbon calibration standard and regular blank checks were done using deionized water (Milli-Q). The error was found to be <1% with an R^2 value of 0.9999, at intercept zero. All measurements were made in replicates. Inorganic anions (Cl^-, and SO_4^{2-}) were measured using a Dionex ICS-1100 Ion chromatography (IC) system (Thermofisher Scientific, USA) with a DS6 heated conductivity cell detector. A mixture of sodium carbonate (Na_2CO_3) and sodium bicarbonate ($NaHCO_3$) was used as the eluent and the sample injection volume was 25 µL as per the instrument protocol. The water samples were filtered using 0.2µm syringe filter before the injection into the IC.

Trace elements present in the samples were analyzed by Inductively coupled plasma mass spectrometry, ICP-MS (Thermo iCAP Q ICP-MS with CETAC ASX-520 autosampler, Thermofisher Scientific, USA). Continuous calibration was performed with standard and blank solutions during measurements. Standard solutions were prepared in 1% HNO_3 using ICP-multielement standard (30 elements) solution VI (Merck Millipore, Germany). The sample preparation was done following Yuan et al. (2004) and Link et al. (1998). The fjord water samples were diluted 25 times to avoid interference with the salts while the snow, ice, and MW samples were taken without dilution.

3(a). 2.4. Total microbial counts and isolation of heterotrophic bacteria

For the analysis of total bacterial cell counts, water samples (10mL) collected from all sampling locations were fixed with buffered formaldehyde (2% final concentration; composition (for 1L): NaH_2PO_4 - 4g, Na_2HPO_4 - 6.5g, commercial formaldehyde - 100mL, NaH_2PO_4 - 4g, Na_2HPO_4 - 6.5g, commercial formaldehyde - 100mL, MilliQ - 900mL, pH - 7.2 ± 0.5) and stored in dark at 4 °C. Total microbial counts in the samples were enumerated using a modified method of Porter and Feig (1980) after staining with 4, 6-diamidino-2- phenylindole (DAPI) (DAPI, 20 µL of 1 µg ml^{-1} working solution per ml of Phosphate buffered saline (PBS); PBS composition (for 1L): NaCl - 8g, KCl - 0.2g, Na_2HPO_4 - 1.44g, KH_2PO_4 - 0.24g, $CaCl_2.2H_2O$ - 0.133g, $MgCl_2.6H_2O$ - 0.10g, pH - 7.4). The stained cells were filtered through black 0.22 µm-pore-size polycarbonate membrane filters (Nucleopore Track-Etch Membrane, Whatman, Maidstone, UK) and counted using Leica DM6 B microscope (Leica Microsystems, Wetzlar, Germany) with the aid of the DAPI filter (Excitation 325–375 nm and Emission 435-485 nm). Counts were made from a minimum of 20 randomly chosen microscopic fields from each sample to obtain a reliable mean (Porter and Feig 1980). Culturable heterotrophic bacteria from the glacier snow, ice, MWs, and fjord samples were enumerated by the spread plate method (Sanders, 2012). To obtain a broad range of bacteria, we used several media in our study based on previous reports of successful recovery and isolation of Polar bacteria (Reddy et al., 2000; Amato et al., 2007; Antony et al., 2016). A 100 µL of sample suspension was directly plated onto solid R2A media (1/2 strength), Antarctic Bacterial Medium (ABM), Tryptone Soy Agar (TSA) ($1/10^{th}$ strength), Luria Bertani medium (LB), Actinomycete isolation agar (AIA), Zobell Marine Agar (ZMA) medium ($1/4^{th}$ strength) and Artificial Seawater (ASW) salts broth agar medium. All media were purchased from Himedia, India. The plates were incubated at 4 °C and 20 °C for 2 - 3 weeks. Colonies with unique morphological features were isolated and sub-cultured to obtain pure cultures.

3(a). 2.5. Rep-PCR analysis, PCR amplification of the 16S rRNA gene, sequencing and phylogenetic analysis

Total bacterial genomic DNA was extracted from the pure bacterial cultures using ChargeSwitch gDNA mini bacteria kit (Invitrogen, Carlsbad, CA, USA). The purity of the DNA extracts was verified by gel electrophoresis on 1% agarose. Repetitive element sequence-based PCR (Rep-PCR) amplification based fingerprinting technique was used with the BOX A1R primer (5'- CTACGGCAAGGCGACGCTGACG-3') to eliminate bacterial species subtypes and to select unique bacterial isolates for further studies (Rademaker et al., 1998) (**Fig. 3.4**). Rep-PCR was carried out in a final reaction volume of 25 µL, containing, 0.3 pM/µL of primer, 12.5 µL of AmpliTaq Gold 360 Master Mix (Applied Biosystems, CA, USA), and 2.5 µL of 360 GC enhancer (Applied Biosystems, CA, USA) and template DNA with a concentration of ~20ng/µL in a thermocycler (BioRad CFX 96, Applied Biosystems, USA) with the following conditions: initial denaturation at 95 °C for 7 min followed by 29 cycles of 1 min at 94 °C, 1 min at 53 ˚C and 8 min at 72 °C, and a final extension of 10 min at 72 °C. Amplification was confirmed by electrophoresis in 2% agarose gel (Yang and Yen, 2012). Isolates with unique banding patterns in the Rep-PCR gel were further chosen for 16S rRNA gene amplification and sequencing as per Sinha et al (2017a) with slight modification. In brief, the 16S rRNA gene was amplified using the universal bacterial 16S primers;

8F:
5'- AGAGTTTGATCCTGGCTCAG -3'
1510R (reverse):
5'- GGTTACCTTGTTACGACTT -3'

The PCR amplification was carried out in a final reaction volume of 25µL containing 0.3 pM/µL of each primer (from 100pM stock) and 12.5 µL of Hi-throughput EmeraldAmp GT PCR mastermix (Takara, Japan) in a thermocycler (BioRad CFX 96) with the following conditions: initial denaturation at 95 °C for 5 min followed by 29

cycles of 1 min at 95 °C, 1 min at 59 °C and 2 min at 72 °C, and a final extension of 10 min at 72 °C. The amplicons thus obtained were purified using the ExoSAP-IT PCR product cleanup reagent (Thermofisher Scientific, USA) and further sequenced using an automated DNA sequencing system (Applied Biosystems 3500 Genetic analyser, Thermofisher Scientific, USA). The obtained sequences were assembled using BioEdit sequence alignment editor version 7.0.5.3 (Hall, 1999) and the obtained consensus contigs were screened for chimera sequences using the USEARCH version 6.0 (http://rdp8.cme.msu.edu/cgis/chimera.cgi) and the chimeric sequences were removed from the further analysis. The 16S rRNA gene nucleotide sequences having greater than 1200 bp were further analyzed in the EZbiocloud server to identify their phylogenetic affiliation against already known validly published type strain species (Yoon et al., 2017). A total of 172 identified 16S rRNA gene sequences belonging to the isolated unique strains were deposited in GenBank under the accession numbers **MH478303-MH478317, MH478329-MH478331, MH478333-MH478335, MH478337-MH478341, MH478345-MH478347, MH482212-MH482349, and MK367606-MK367610**.

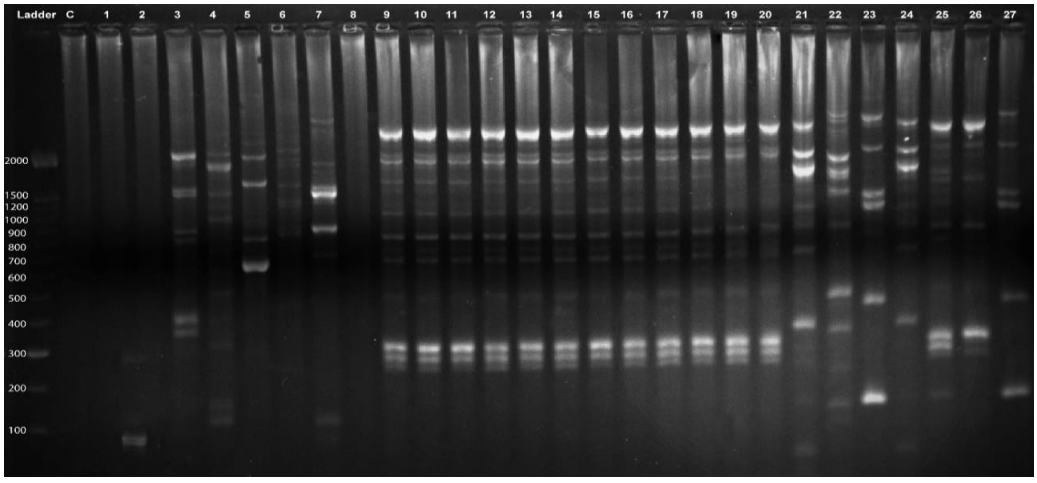

Fig. 3.4. Fingerprint patterns of the Rep PCR gel image observed for MW isolates. The first lane from the left represents the molecular ladder, C- represents negative control, Lanes marked 1 - 27 represents the MW isolates.

3(a). 2.6. DNA extraction and metagenomics library preparation for amplicon sequencing

Total DNA was extracted from the previously frozen 0.22 µm pore-size polycarbonate membranes filters using a DNeasy Power Soil kit (Qiagen, USA) according to the manufacturer's protocol. The quality of the eluted DNA samples was checked on gel electrophoresis by using 1% agarose gel and the DNA concentration was measured using the Qubit fluorometer (Thermo Fisher Scientific, USA). The DNA samples were subjected to amplification of the V3-V4 region of the bacterial 16SrRNA gene using Pro341F/Pro805R primers (Takahashi et al., 2014).

Pro341F

5' - CCTACGGGNBGCASCAG - 3'

Pro805R

5'- GACTACNVGGGTATCTAATCC - 3'

The PCR reaction conditions for DNA amplification were as follows: initial denaturation at 98 °C for 2 min, followed by 35 cycles of annealing starting at 65 °C and ending at 55 °C for 15 sec, and extension at 68 °C for 30 sec. The annealing temperature was lowered 1 °C every cycle until reaching 55 °C, which was used for the remaining cycles (Touch down PCR method, Takahashi et al., 2014). The amplified products were added with multiplex identifier tags and template-specific nucleotide primers for each sample. Sequencing was carried out using a paired end 2×250bp cycle run on an Illumina HiSeq sequencing platform 2500 (Illumina, USA). The raw DNA nucleotide sequence datasets were deposited into the NCBI Sequence Read Archive (SRA) database under the accession number: **SRP150318**.

3(a). 2.7. DNA sequence data curation and bioinformatics analysis

Further downstream processing of raw DNA sequence datasets was carried out by the methods described in Caporaso et al. (2010) with the following modifications. Sequences belonging to 16S rRNA gene primers and multiplexing adapters were removed from the raw sequence datasets by using Cutadapt (v.1.18) software (Martin, 2011). The quality of the reads was further checked using the FastQC program followed by merging the paired-end reads using the Seqprep program (v.1.3.2) (https://github.com/jstjohn/SeqPrep). The merged sequences were pooled and clustered into Operational Taxonomic Units (OTUs) using the open reference OTU picking protocol (Rideout et al., 2014). The clustering of reads into OTUs (at 97% sequence similarity) was performed against the Greengene reference database (v.13_5) using the Uclust program. In this step, singleton OTUs as well as OTUs whose representative (i.e., centroid) sequence couldn't be aligned with PyNAST were removed. Unique representative OTUs from each sample were further subjected to the taxonomic classification. OTUs representing the chloroplasts and mitochondrial sequences were removed from the analysis by using QIIME software (Caporaso et al., 2010). The OTU counts were rarefied (10 iterations) and various alpha diversity indices were calculated using Shannon, Simpson, Chao1, and observed species metrics in QIIME software.

3(a). 2.8. Statistical analysis

Double hierarchial dendrogram and the heatmap depicting the relative abundance of the top 100 bacterial OTUs within the 13 samples were plotted using the multivariate statistical software Primer (PRIMER E) version 7. Venny 2.1 software (Oliveros, 2007) was used to generate a Venn diagram to demonstrate the number of site-specific OTUs and the common OTUs shared between the sites. Analysis of similarities (ANOSIM) was performed to test the significance of the differences observed between the three sampling sites (VB glacier, MWs, and fjord) using PRIMER 7 software. Similarity percentage (SIMPER) analysis was used to calculate the percent contribution of bacterial OTUs to the average dissimilarity between the sampling sites (Clarke et al.,

1993) and one-way analysis of variance (ANOVA) test was performed to determine the significant environmental parameters influencing the sampling sites using PAST (v.3.21) software (Hammer et al., 2001). The relevance of environmental factors in explaining the distribution patterns of bacterial communities in different sampling sites was done by Bray - Curtis distance-based Redundancy analysis (dB-RDA) using the vegan package in R v.3.1.1 statistical software.

3(a). 3. Results

3(a). 3.1. Environmental characteristics of the glacier, meltwaters, and fjord waters

The sampling locations and the various environmental parameters analyzed in the study are summarized in **Table 3.1**. The highest average concentrations of NO_3^- (5.65 µM) and SiO_4^{2-} (6.53 µM) were observed in the MWs as compared to the glacier snow, glacier ice, and fjord waters while the highest values for NO_2^- (0.51 µM) and PO_4^{3-} (1.48 µM) were found in the glacier ice. The maximum average concentration of TOC was observed in the Kongsfjorden waters (25.6 ± 4 ppm). MWs had an average TOC value of 0.84 ± 0.2 ppm while the glacier ice had a TOC value of 0.59 ppm. The glacier snow sample VBSS1 had a TOC concentration of 0.71 ppm while the snow sample VBSS2 had a TOC value of 0.83 ppm. The highest average concentrations of Cl^- (16.18 ± 6.4 ppt) and SO_4^{2-} (2.14 ± 0.9 ppt) ions were found in the fjord waters. The average concentrations of Cl^- and SO_4^{2-} ions in the MWs were 5.91 ± 3.9 ppm and 16.47 ± 3.9 ppm while in the glacier snow, it was found to be 5.9 ± 0.1 ppm and 1.48 ± 0.2 ppm respectively. The highest concentrations of trace metals such as Mn, Fe, Ni, and Cu were observed in the Kongsfjorden waters as compared to the glacier snow, ice, and MWs. Among the Kongsfjorden samples, the inner fjord water sample KNS10 was found to exhibit the highest concentrations of Mn (25.11 µg/Kg), Fe (249.9 µg/Kg), and Ni (24.39 µg/Kg). VB snow sample (VBSS1) exhibited the highest concentration of Zn (566.6 µg/Kg) and Co (3.64 µg/Kg) as compared to the MWs and fjord water samples (**Table 3.2**). It was observed that except PO_4^{3-} and NO_2^-, all other parameters such as NO_3^-, SiO_4^{2-}, Cl^-, SO_4^{2-}, and the trace metals, contributed to significant

differences between the glacier (snow and ice), MWs, and fjord samples (ANOVA, $p < 0.05$) (**Table 3.3).**

3(a). 3.2. Bacterial enumeration and identification of culturable bacteria

Total microbial count (TMC) observed in the samples ranged between 10^{4-8} cells/L with the lowest (10^4 cells/L) and highest count (10^8 cells/L) recorded in the snow samples and Kongsfjorden waters respectively. A total bacterial count of 10^5 cells/L could be observed in the glacier snout ice while 10^7 cells/L were recorded in the MWs (**Table 3.1**).

The total retrievable heterotrophic bacterial count in the Kongsfjorden waters was found to be 10^6 CFU/L while MWs shown to have a total retrievable count within the range of 10^{4-6}, which was followed by the VB glacier ice sample (10^3 CFU/L) (**Table 3.1**). The total retrievable heterotrophic bacterial count for the VB glacier ice and MW samples were calculated using the colony counts obtained on TSA plates ($1/10^{th}$ strength) while for the Kongsfjorden total retrievable count, ZMA plates ($1/4^{th}$ strength) were used. We were unable to retrieve bacteria from the VB snow even after enrichment in broth media for a prolonged period of incubation (3 months).

A total of 20 (11 from 4 °C and 9 from 20 °C incubation), 510 (278 from 4 °C and 232 from 20 °C incubation), and 180 (114 from 4 °C and 66 from 20 °C) bacterial isolates were recovered from glacier ice, MWs, and Kongsfjorden waters respectively. Based on fingerprint profile generated by rep-PCR, a total of 20 isolates from the glacier ice (11 isolates from 4 °C and 9 isolates from 20 °C incubation), 214 from the MWs (129 isolates from 4 °C and 85 isolates from 20 °C incubation) and 34 isolates from the Kongsfjorden waters (14 isolates from 4 °C and 20 isolates from 20 °C incubation) were selected for 16SrRNA sequencing. The 16S rRNA gene sequence data analysis revealed the taxonomic grouping of bacterial isolates into 5 species from the glacier ice, 40 species from the MWs, and 16 species from the Kongsfjorden waters (similarity cutoff > 98.7%; Kim et al., 2014) (**Table 3.4**). Seventeen bacterial isolates that were retrieved from the glacier ice, and MWs showed < 98.7% similarity with the closest phylogenetic neighbor and hence might represent novel taxa (**Table 3.4**).

The bacterial isolates retrieved from the glacier ice, MWs as well as from the fjord waters belonged to three phyla namely, Proteobacteria (with the classes α, β, and γ-proteobacteria), Bacteroidetes, and Actinobacteria. Proteobacteria was found to be the most frequently encountered phyla in the glacier ice (66.6% of the total isolates), MWs (82%), and fjord (97%) waters, among which α- proteobacterial class dominated the VB ice (75% of the total proteobacteria) while γ-proteobacterial class dominated the MW samples (61.25% of total proteobacteria) and fjord water samples (87.6%) (**Fig. 3.5**). In the VB ice, β-proteobacteria contributed to 25% of the total proteobacterial population while in MWs their contribution accounted for 31.6%. In the fjord water samples, β-proteobacteria could not be recovered. The other phyla, namely Actinobacteria was represented by 8.33%, 8.62%, and 2% of the total isolates in glacier ice, MWs, and fjord samples, while Bacteroidetes represented 25%, 9.35%, and 0.6% of the total respectively (**Fig. 3.5**).

The culture medium TSA ($1/10^{th}$ strength) and R2A (1/2 strength) yielded maximum retrieval of colonies from the glacier ice and MW samples as compared to the other media used in the study (**Table 3.5**). It was also noted that the majority of the isolates retrieved from the glacier ice and MW samples were pigmented (orange, red, pink, peach, and yellow). Among the VB ice isolates, *Polymorphobacter glacialis* (Class α-proteobacteria) was found to be the dominant species (37.5% of the total isolates), followed by *Flavobacterium piscis* belonging to Class Flavobacteriia (16.6%) and *Herminiimonas fonticola* belonging to Class β-proteobacteria (14.5%). The Actinobacteria phylum was represented by a single genus *Cryobacterium* with 3 different species namely - *C. psychrotolerans, C. flavum,* and *C. levicorallinum*. While, in the MWs, *Pseudomonas extremaustralis* (48% of the total) was found to be the most abundant bacterial species along with substantial contributions from *Polaromonas* (19.7% of the total) and *Flavobacterium* (8.6%). In the MWs, class γ-proteobacteria was represented by a single genus, *Pseudomonas* while a total of 7 genera were recovered within the class α-proteobacteria wherein the species *Sphingorhabdus planktonica* was the major contributor (38% of α-proteobacteria). β-proteobacteria was represented by 7 genera among which *Polaromonas eurypsychrophila* was the predominant species (72% of β-proteobacteria). Three genera represented the phylum

Bacteroidetes wherein the major fraction was contributed by the genus *Flavobacterium* (98% of Bacteroidetes).

Phylum Actinobacteria was represented by 13 different genera, of which *Glaciihabitans* and *Cryobacterium* appeared to be most dominant (**Table 3.4**). Of all the isolates identified from the VB glacier ice and MWs, 52% isolates were retrieved by incubating the samples at 4 °C, 23% isolates were from 20 °C incubation, while 25% of isolates identified had their representatives at both temperature incubations.

Table 3.1.

Details of sampling locations and their associated physicochemical parameters measured in the study.

Parameters	Glacier			Melt Water Channels						Kongsfjorden			
	VBSS1	VBSS2	VB Ice	MW1	MW2	MW3	MW4	MW5	MW6	KNBR (DCM=10m)	KNS1 (DCM=30m)	KNS9 (DCM=10m)	KNS10 (DCM=10m)
Latitude Longitude	78.8945°N 11.6695°E	78.9114°N 11.7274°E	78.91375°N 11.77069°E	78.91761°N 11.81048°E	78.9164°N 11.77842°E	78.92436°N 11.79769°E	78.92436°N 11.80883°E	78.92678°N 11.82803°E	78.93249°N 11.83587°E	78.93472222°N 11.905277°E	79.0354°N 11.2836°E	78.8951°N 12.3201°E	78.9931°N 12.3°E
Temperature (◦C)	-6.2	-13.2	1	0.9	1.9	1.3	0.4	1.3	0.9	4.5	6	4.2	3.2
DO(mg/L)	ND	ND	ND	7.82	8.9	8.01	7.8	7.711	7.92	8.5	6.75	5.6	6
Total Dissolved Salts(TDS)/ Salinity (ppm for MW, ppt for fjord)	ND	ND	ND	81.39	24.58	107.8	109.7	87.19	93.43	33	34.5	33	31
Nitrite (µM)	0.09	0.08	1.35	0.27	0.09	0.13	0.08	0.84	0.63	0.08	0.01	0.05	0.1
Nitrate (µM)	0.08	0.08	1.3	4.6	1.3	8.98	7.93	5.37	5.74	0.62	0.11	0.43	0.56
Total Phosphate (µM)	0.25	0.21	3.97	0.22	0.25	0.29	0.24	1.95	1.66	0.27	0.2	0.22	0.17
Silicate (µM)	0.41	0.44	4.61	6.66	4.61	8.67	4.19	6.72	8.31	0.83	0.64	0.75	0.79
Chloride (ppm for Glacier and MW, ppt for fjord)	5.82	5.99	2.08	2.91	9.99	2.59	11.3	6.14	2.55	18.11	19.6	20.4	6.65
Sulphate (ppm for Glacier and MW, ppt for fjord)	1.63	1.32	*	11.2	*	21.75	14.81	16.44	18.16	3.14	2.16	2.33	0.93
TOC (ppm)	0.71	0.83	0.59	0.52	0.89	0.71	0.92	0.88	1.1	23.8	25.7	31.1	21.7
TMC (cells/L)	2.2×10^4	3.4×10^4	5.4×10^5	6.2×10^7	7.5×10^7	7.4×10^7	6.7×10^7	6.3×10^7	6.5×10^7	5.5×10^8	7.2×10^8	6.1×10^8	6.6×10^8
RHBC 4°C (CFU/L)	ND	ND	9×10^3	3.6×10^5	7×10^4	5.9×10^5	5×10^5	1×10^4	7×10^5	3.8×10^6	7.3×10^6	4.3×10^6	7.6×10^6
RHBC 20°C (CFU/L)	ND	ND	6×10^3	3.16×10^5	5×10^4	3.7×10^5	3.9×10^5	2.6×10^4	1×10^6	1.6×10^6	4.6×10^6	3.3×10^6	6×10^6

**below the detection limit, ND- Not Determined, DCM – Deep Chlorophyll Maximum*

Table 3.2.
Details of the trace element concentration from various sampling sites.

Station Details	Mn (µg/Kg)	Fe (µg/Kg)	Co (µg/Kg)	Ni (µg/Kg)	Cu (µg/Kg)	Zn (µg/Kg)
VBSS1	6.32	23.08	3.64	0.40	0.12	566.6
VBSS2	4.93	10.67	1.88	3.92	0.14	421.67
VB ICE	0.59	3.37	0.02	0.37	0.16	7.77
MW1	0.08	0.44	0.01	0.03	0.19	1.04
MW2	0.55	2.08	0.01	0.16	0.04	9.47
MW3	0.94	7.99	0.01	1.85	0.22	18.81
MW4	0.36	4.69	0.04	0.19	0.13	9.98
MW5	2.32	3.73	0.03	0.16	0.12	4.46
MW6	1.15	3.36	0.02	0.36	0.11	2.6
KNBR	10.14	105.14	0.79	9.99	3.3	321.89
KNS 1	8.71	75.32	0.68	6.8	6.31	355.22
KNS 9	8.41	113.85	0.32	5.55	3.66	483.72
KNS 10	25.11	249.9	0.99	24.39	3.19	84.09

Table 3.3.
One way analysis of variance (ANOVA) F-value and p-value for the various chemical parameters.

	F value	p value
Anions		
Chloride	29.29	6.59E-05
Sulphate	25.21	0.000124
Nutrients		
Nitrate	11.63	0.002457
Silicate	16.27	0.000717
Phosphate	1.08	0.3752
Nitrite	1.16	0.3526
Trace elements		
Mn	8.46	0.007079
Fe	12.70	0.001799
Co	4.87	0.033325
Ni	6.96	0.01279
Cu	33.44	3.72E-05
Zn	6.28	0.01714

The critical value of F for the one-way ANOVA is 4.102821. For the p-values <0.05 and F-value > F crit (critical value of F), there exists a significant difference between the sampling groups (VB glacier, MWs, and Kongsfjorden waters).

For the Kongsfjorden water samples, maximum retrieval of colonies could be observed on ZMA (1/4th strength) media (**Table 3.5**). From the culture-dependent study in the Kongsfjorden waters, we could observe the highest relative abundance of *Alteromonas stellipolaris* (58.8% of the total), along with significant contributions from *Psychrobacter* (10.3%) and *Loktanella* sp. (7%). A total of 5 genera were recovered within the class γ-proteobacteria wherein *Alteromonas stellipolaris* (69% of γ-proteobacteria) was the major contributor. Among the species representing α-proteobacteria, *Loktanella salsilacus* was found to be the dominant candidate (59% of α-proteobacteria). The Actinobacteria candidates belonged to 3 different genera while the phylum Bacteroidetes was represented by two genera. Two bacterial species, closely related to *Flavobacterium degerlachei* and *Salinibacterium xinjiangense* were retrieved from MWs as well as fjord samples (**Table 3.4**). Among the culturable isolates identified from Kongsfjorden waters, 40% of the isolates identified were retrieved by incubating the samples at 4 °C incubation, 47% isolates were isolated from 20 °C incubation, while 13% isolates identified had their representatives at both temperature incubations.

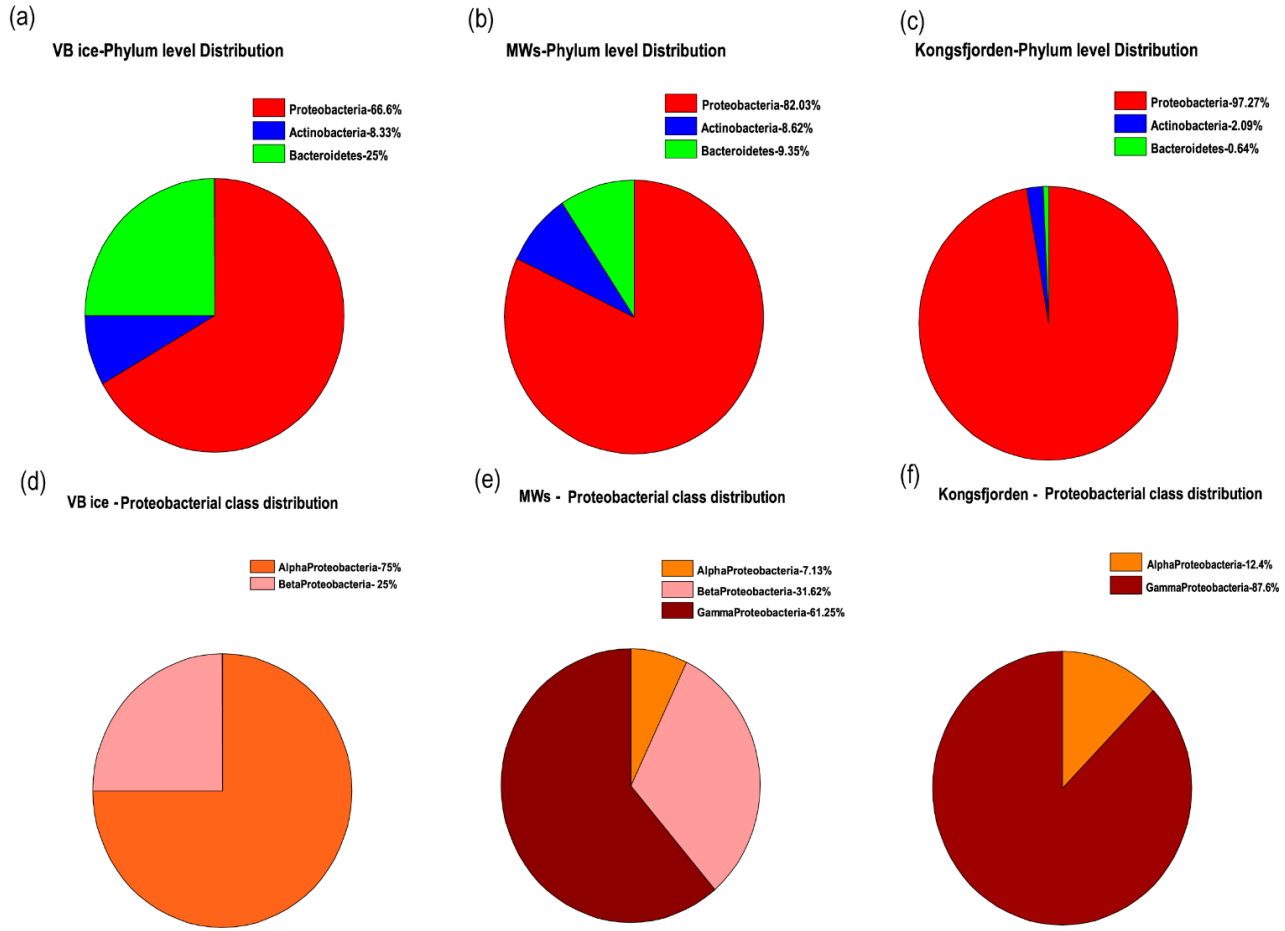

Fig. 3.5. Percentage composition and abundance of retrievable bacteria belonging to the phyla Proteobacteria, Actinobacteria, and Bacteroidetes from (a) VB ice, (b) MWs as well as from (c) Kongsfjorden waters. The phylum proteobacteria which was found to be the dominant phylum among all the sampling sites were then further represented at their class level and the percentage abundance of each of the proteobacterial classes for the (d) VB ice, (e) MWs as well as for the (f) Kongsfjorden was plotted.

Table 3.4.

List of retrievable bacterial isolates from VB glacier, MWs, and Kongsfjorden with their taxonomic affiliation with type strain description. Sequences with similarity levels < 98.7% mostly likely represent novel species that are underlined.

Strain ID	Closest relative type strain	% similarity	Isolation source of type strain/description
	VB glacier ice & MWs		
20VBT1, 4ABL22 (1,2)	*Herminiimonas fonticola (AY676462)*	97.18	Genus nov. Species nov. Isolated from the source of bottled mineral water, Portugal
20VBT2, 4VBT2, 20VBR5 (1,3)	*Polymorphobacter glacialis* (KP013180)*	98.89	From ice core of Muztag glacier, Tibetan Plateau
20VBR2, 20VBT3 (1,3)	*Porphyrobacter sanguineus*(FRDF01000003)*	99.25	Bacteriochlorophyll containing marine bacteria
20VBT4 (1)	*Paucibacter toxinivorans (AY515390)*	98.56	Genus nov. Species nov. cyanobacterial toxin-producing bacteria from lake sediment
20VBR1 (3)	*Phenylobacterium aquaticum*(KT309087)*	97.85	From the reservoir of a water purifier
4BR6T24, 4BR6A10, 20BR6T6 (1,4)	*Aquaspirillum arcticum* (AB074523)*	97.83	Genus nov. Species nov. From Antarctic coastal water
4ABA6 (4)	*Alpinimonas psychrophila* (GU784868)*	100	From the Alpine glacier cryoconite, Rettenbach glacier, Austria
4ABAA1, 4BR4Z17, 4BR4A5, 20BR4Z8, 20BR4AA10 (4,5,6)	*Arthrobacter ginsengisoli (KF212463)*	98.77	From the soil of a ginseng field, the Republic of Korea
20BR4R14,4BR4Z16 (3,6)	*Arthrobacter psychrochitiniphilus (AJ810896)*	99.23	From Adelie penguin guano from Antarctica
4ABZ3 (6)	*Brevibacterium frigoritolerans (AM747813)*	99.86	From sugarcane fields soil in Gurdaspur district of Punjab, India
20BR2R2 (3)	*Brevibacterium pityocampae (EU484189)*	99.62	From larvae of the pine processionary moth
4BR4R27 (3)	*Brevundimonas staleyi*(AJ227798)*	98.85	From activated sludge
4BR6L11, 4BR6T21, 20BR6R10 (1,2,3)	*Brevundimonas subvibrioides*(ADBM01000034)*	99.25	From Pond water

4ABR23, 20BR4A1, 20BR4R21, 4ABL27, 4BR4A7 (2,3,4)	*Caulobacter henricii (AJ227758)*	99.7	From Freshwater lake
4BR6A9 (4)	*Clavibacter michiganensis subsp. michiganensis* (jgi.1118350)*	97.37	Plant pathogen infecting tomato
4BR6R17, 4BR6R1 (3)	*Compostimonas suwonensis* (JN000316)*	97.4	From Spent mushroom compost sample in Suwon, S.Korea
4BR6T29, 4BR6T5, 4VBT8 (1)	*Cryobacterium flavum (jgi.1076268)*	100	From No. 1 glacier in Xinjiang, China
4BR4R8, 4BR4AA1, 4BR4R14, 4VBA1 (3,4,5)	*Cryobacterium levicorallinum (JF267312)*	99.93	From No. 1 glacier in Xinjiang, China
4BR4Z13, 4BR6L18 (2,6)	*Cryobacterium luteum (jgi.1076264)*	99.86	From No. 1 glacier in Xinjiang, China
4VBA2, 4ABAA7, 4ABL30, 4ABR41, 4ABT14 (1,2,3,4,5)	*Cryobacterium psychrotolerans(jgi.1076200)*	98.26	From No. 1 glacier in Xinjiang, China
4BR6L5, 4BR4AA2 (2,5)	*Cryobacterium roopkundense (EF467640)*	99.35	From Glacial Lake in Roopkund, Himalayan mountain range
4BR4T9, 20BR4R16 (1,3)	*Duganella phyllosphaerae*(FR852575)*	97.49	From leaf surface of Trifolium plant
4BR4T1, 4BR4R21, 4BR4R3, 4BR4R10, 20BR4R23 (1,3)	*Flavobacterium degerlachei (jgi.1107974)*	99.27	From Microbial mats in Antarctic lakes
4ABA2, 4ABR3, 4ABA9, 4ABA19, 4ABA29, 4ABA30, 4ABL28, 4ABR12, 4ABR21, 4ABR37, 4ABZ17, 4ABT6, 4ABT5, 4BR4R17, 4BR4R13 (1,2,3,4,6)	*Flavobacterium fryxellicola (jgi.1107689)*	98.11	From Microbial mats in Antarctic lakes
4ABT9, 4ABR31 (1,3)	*Flavobacterium granuli (jgi.1107693)*	98.26	From No. 1 glacier in Xinjiang, China
4ABT20, 4ABR5 (1,3)	*Flavobacterium omnivorum (AF433174)*	98.08	From No. 1 glacier in Xinjiang, China
4BR4R28 (3)	*Flavobacterium pectinovorum (jgi.1107681)*	97.75	From the soil in southeast England
4BR4Z2, 4VBT5 (1,6)	*Flavobacterium piscis (LVEN01000016)*	97.85	From diseased rainbow trout
4ABR28, 4ABR29 (3)	*Flavobacterium psychrolimnae (AJ585428)*	98.48	From microbial mats in Antarctic lakes
4BR4A8, 4ABR2, 4ABR14 (3,4)	*Flavobacterium sinopsychrotolerans (FJ654474)*	99.85	From frozen soil from the No. 1 glacier, China
4ABA24 (4)	*Flavobacterium xinjiangense (AF433173)*	99.56	From No. 1 glacier in Xinjiang, China

4ABT8 (1)	*Flavobacterium xueshanense (HQ436466)*	99.13	From water of melted ice from the No.1 glacier, China
4BR6L2, 4BR6R11, 4BR2R2, 4BR6Z2, 4BR6Z10, 4BR6AA1, 4BR6AA10, 4BR6A3, 4BR6A6, 4BR6T10, 4BR6T16 (1,2,3,4,5,6)	*Glaciihabitans tibetensis*(KC256953)*	97.73	From ice water of Midui glacier, Tibet
20ABR8, 20ABL2 (2,3)	*Hydrogenophaga palleronii (BCTJ01000079)*	99.14	Yellow-pigmented hydrogen-oxidizing species
4BR6R7 (3)	*Hymenobacter frigidus (KJ755878)*	98.38	From Victoria glacier, Antarctica
20BR2R1 (3)	*Janibacter alkaliphilus*(JN160681)*	100	From a gorgonian coral sample of Anthogorgia sp.
20BR4R40, 4BR4L3, 20BR4L12, 20BR4L16, 20BR4R46 (2,3)	*Janthinobacterium svalbardensis (DQ355146)*	99.86	From glacier ice samples from the island Spitsbergen in the Arctic
4ABZ16 (6)	*Leifsonia rubra* (ATIA01000019)*	99.35	From a cyanobacterial mat from Antarctic pond
4BR2R1, 4BR6R10, 4ABZ15, 4ABT3, 4ABT16, 20BR6Z1 (1,3,6)	*Marisediminicola antarctica*(GQ496083)*	99.93	From sandy intertidal sediment sample from east Antarctica
4BR6R2, 4BR6T2, 4ABR7, 4ABT17, 20BR6R19, 20ABR10, 20ABT6, 20ABT8, 20ABT4, 20ABL8, 20ABR10, 20BR6R17, 20BR6T2, 20BR6T3 (1,2,3)	*Polaromonas eurypsychrophila (KP013181)*	98.28	From Ice core from Muztagh Glacier on the Tibetan Plateau, China
4ABT2, 4ABL13, 4ABR20 (1,2,3)	*Polaromonas glacialis (HM583568)*	99.07	From the Alpine glacier cryoconite, Austria
4BR6T27 (1)	*Polymorphobacter fuscus* (KF737330)*	98.73	Bacteriochlorophyll *a*-containing bacterium from permafrost soil of Kunlun mountains gap, Qinghai-Tibet plateau
4BR6T11 (1)	*Polymorphobacter glacialis*(KP013180)*	98.82	From Ice core from Muztagh Glacier on the Tibetan Plateau, China.
4ABA27, 4ABL1, 20BR4A3 (2,4)	*Pseudarthrobacter oxydans (X83408)*	98.68	Nicotinophilic bacteria. Isolated from Arctic Tundra and reported for Hg tolerance
4ABT11 (1)	*Pseudarthrobacter scleromae (AF330692)*	98.76	From swollen scleromata of a dermatosis patient
20ABA5, 20ABL3 (2,4)	*Pseudarthrobacter sulfonivorans (AF235091)*	99.12	Facultative methylotrophs. Reported for degradation of crude oil and multibenzene compounds
4BR5AA2, 4BR5Z8, 4BR5A3, 20BR5A9, 20BR5AA7 (4,5,6)	*Pseudomonas avellanae (AKBS01001374)*	99.22	Causing canker of hazelnut

4BR5Z1, 4BR1R10, 4BR5Z3, 4BR5A2, 4BR4AA5, 4BR4R36, 20BR5AA14, 20BR5AA10, 20BR5L3, 20BR5R15, 20BR4Z3, 20BR4Z15, 20BR4AA9, 20BR4R12, 20BR4R20, 20BR5R28, 20BR5R30, 20BR4VA1, 20BR1VA1, 20BR5VA1 (3,4,5,6)	*Pseudomonas extremaustralis (AHIP01000073)*	99.64	PHB producer from Antarctic pond
4BR4Z1 (6)	*Pseudomonas kilonensis (LHVH01000037)*	99.09	From agricultural soil from northern Germany
20BR4AA8, 4ABA14, 4BR5T4, 4BR5R1, 20BR5T16, 20BR5AA9, 20BR5R20, 20BR6R17, 20BR4L5, 20BR4AA12, 20BR4R35, 20BR4A7(1,2,3,4,5)	*Pseudomonas mandelii (AF058286)*	98.86	From natural mineral waters
20BR4VA2(4)	*Pseudomonas orientalis (AF064457)*	99.71	From Lebanese spring waters
4BR5T2 (1)	*Pseudomonas trivialis (JYLK01000002)*	99.28	Fluorescent pseudomonads, isolated from the phyllosphere of grasses
4BR6R13, 4BR6L7 (2,3)	*Salinibacterium xinjiangense (DQ515964)*	99.49	From No. 1 glacier in Xinjiang, China
20ABL1 (2)	*Sphingomonas faeni (AJ429239)*	99.47	From cow-barn air
20ABL7 (2)	*Sphingomonas ginsenosidimutans (NWVD01000032)*	100	From the soil of ginseng cultivating field, South Korea
20ABR2, 20ABR11, 20ABA1 (3,4)	*Sphingomonas paucimobilis (BBJS01000072)*	100	Opportunistic pathogens
20BR6R1, 4BR6R18, 20BR6R6, 20BR6R8, 20ABR13, 20BR6R14 (3)	*Sphingorhabdus planktonica*(JN381068)*	99.78	From oligotrophic freshwater lake in Bavaria, Germany
20BR6R7, 20BR6R12, 20BR6R13, 20BR6R12 (3)	*Sphingorhabdus wooponensis*(HQ436493)*	99.63	From Woopo wetland, S.Korea
20BR3R10 (3)	*Ponticoccus gilvus* (AM980985)*	100	From seawater sample in Republic of Korea
	Kongsfjorden		
4KNS10Z2 (6)	*Ahrensia kielensis* (ARFW01000003)*	99.69	From Sea water of Baltic sea
20KNS1ASW1, 20KNS1Z2, 20KNS1Z4, 20KNS10Z2 (6,7)	*Alteromonas stellipolaris (CP013926)*	99.71	From Antarctic seawater
4KNBRT7 (1)	*Flavobacterium degerlachei (jgi.1107974)*	99.46	From microbial mats in Antarctic lakes

20KNS1T2 (1)	*Kocuria palustris (Y16263)*	99.93	From rhizoplane of the narrow-leaved cattail
4KNBRR5 (3)	*Loktanella salsilacus (jgi.1058898)*	99.85	From microbial mats in Antarctic lakes
4KNBRZ7 (6)	*Maribacter arcticus (jgi.1049076)*	99.71	From marine sediment from Ny Alesund, Spitsbergen, Norway
4KNSF6T1 (1)	*Paraglaciecola mesophila* (BAEP01000046)*	99.49	Agarolytic bacteria from marine invertebrates
20KNS10Z3, 20KNS1Z1, 20KNSF6Z1 (6)	*Pseudoalteromonas distincta (JWIG01000030)*	99.93	From Amursky Bay (Sea of Japan, Pacific Ocean)
20KNS1Z5 (6)	*Pseudoalteromonas translucida (CP011034)*	99.57	From Gulf of Peter the Great, sea of Japan
20KNS10Z4, 4KNS1L2, 20KNSF6Z2, 20KNS1Z3, 20KNS10L4, 20KNS10R7 (2,3,6)	*Pseudomonas sabulinigri (LT629763)*	98.79	From black sand, Jeju Island, Korea
20KNBRL6 (2)	*Psychrobacter glaciei (FJ748508)*	99.86	From Ice core of Austre Lovénbreen, Ny-Ålesund, Svalbard
20KNS10L2, 4KNS9A1, 20KNS10L1, 20KNS10T3 (1,2,4)	*Psychrobacter nivimaris (AJ313425)*	99.78	attached to the organic particles, from the South Atlantic (Antarctica)
20KNS10T6 (1)	*Rhodococcus yunnanensis (BCXH01000047)*	99.13	From forest soil sample in Yunnan Province, China, mesophilic bacteria
4KNSF6AA1 (5)	*Salinibacterium xinjiangense ((DQ515964))*	99.34	From No. 1 glacier in Xinjiang, China
4KNBRR6 (3)	*Sphingopyxis baekryungensis (ATUR01000005)*	99.84	From seawater of the yellow sea in Korea
20KNS9L3 (2)	*Sphingorhabdus flavimaris (AY554010)*	99.85	From seawater of the Yellow sea, Korea

** indicates the bacterial strains which were observed only in our culture-dependent study and not observed in the culture-independent work. The superscript numbers in the bracket along with the strain IDs indicate the different media of isolation for each bacterial species (1-Tryptic Soya Agar (1/10 strength), 2-Luria Bertani Agar (1/2 strength), 3-R2A Agar (1/2 strength), 4-Antarctic Bacterial Medium, 5-Actinomycete Isolation Agar, 6-Zobell Marine Agar (1/4 strength), 7-Artificial Sea Water salts agar. The temperature incubation at which the bacteria were isolated is represented by the first 1 or 2 numbers in the bacterial ID. For eg.* ***20****VBR1- 20°C,* ***4****BR6R17- 4°C.*

Table 3.5.
CFU (colony forming unit) count for the different media at different temperature incubations used for the culture-based work.

Stations	**TSA (1/10 strength)**		**R2A (1/2 strength)**		**ABM**		**LB (1/2 strength)**		**25% ZMA**		**AIA**		**ASW Agar**	
(No. of colonies/mL)	**4°C**	**20°C**	**4°C**	**20°C**	**4°C**	**20°C**	**4°C**	**20°C**	**4°C**	**20°C**	**4°C**	**20°C**	**4°C**	**20°C**
VBSS1	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG
VBSS2	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG
VB Ice	9	6	7	5	3	0	0	0	0	0	0	0	0	0
MW1	360	316	162	290	180	60	230	150	110	10	30	0	0	0
MW2	70	50	21	20	40	0	17	0	20	10	30	0	0	0
MW3	590	370	370	300	730	90	50	370	430	0	710	210	0	0
MW4	500	390	340	220	140	50	210	120	140	70	90	50	0	0
MW5	10	26	8	10	0	0	0	0	0	0	0	0	0	0
MW6	700	1100	520	0	30	0	40	0	60	0	60	0	0	0
KNBR	100	58	460	70	0	0	11	20	3800	1600	600	0	0	540
KNS1	170	105	245	90	0	0	20	15	7300	4600	0	0	0	0
KNS9	0	0	0	0	10	0	150	45	4300	3300	120	0	0	0
KNS10	130	270	10	50	0	0	50	160	7560	6000	0	0	0	0
Total retrievable species identified from diff media														
VB Ice and MWs	21	6	20	18	15	7	10	7	12	4	5	4	0	0
Kongsfjorden	2	3	2	1	1	0	1	2	2	4	1	0	0	1

3(a). 3.3. Bacterial diversity and community composition using 16S rRNA gene amplicon sequencing

A total of 49872 OTUs were identified from the total of 3790722 sequences among 13 samples analyzed in the present study. The analyzed sequence datasets also contain three archaeal phyla namely Euryarchaeota, Thaumarchaeota, and Parvarchaeota. Sequences affiliated with phyla Proteobacteria, Bacteroidetes, Verrucomicrobia, and Actinobacteria were common among all the sampling sites.

Irrespective of the sampling sites, Proteobacteria was the dominant phyla in terms of its relative abundance (38.5% - 99.6%), with the glacier snow samples exhibiting the highest abundance (99.6% in VBSS1 and 83.5% in VBSS2) (**Fig. 3.6a**). The glacier ice bacterial community was dominated by the phyla Proteobacteria (41.6%), Bacteroidetes (36.2%), Cyanobacteria (12.8%), and Actinobacteria (5.3%). In the MWs we could observe the abundance of Proteobacteria, Bacteroidetes, Actinobacteria along with the increased presence of Candidate Division TM7 and OD1. The phyla which were found exclusively in the MWs include Caldiserica, Chlamydiae, Candidate Division GN04, Candidate Division BHI80-139, Candidate Division Kazan-3B-28, and Candidate Division FCPU426. In the Kongsfjorden waters, we could observe a marked increase in the OTUs belonging to phylum Verrucomicrobia from the outer to the inner fjord waters (6.25 - 24.35%) which was significantly higher as compared to the glacier and MW samples (**Fig. 3.6a**).

Among the Proteobacterial OTUs, we could observe the dominance of sequences affiliated with β-proteobacteria in the VB glacier snow samples (89.3% in VBSS1 and 33.2% in VBSS2). The α-proteobacterial OTUs represented about 10-16% of the total in both the glacier snow samples while the γ-proteobacterial OTUs exhibited a higher abundance in the VBSS2 snow sample (33.9%) as compared to the VBSS1 snow (0.2%) (**Fig. 3.6b**). The glacier ice community was dominated by β-proteobacteria (19.2%), Flavobacteriia (19.1%), α-proteobacteria (15.6%), Synechococcophycideae (12.2%), and Cytophagia (9.7%) along with significant contributions of Actinobacteria, Sphingobacteriia, and γ-proteobacteria. In the MW samples, the most abundant proteobacterial sequences encountered was β-proteobacteria (24 - 75%), followed by

γ-proteobacteria (0.3 - 21%), α-proteobacteria (2.2 - 9.3%), and δ-proteobacteria (0.5 - 6.5%). The bacterial classes such as Cytophagia, Flavobacteriia, Sphingobacteriia, Actinobacteria, and TM7-1 were in higher abundance in the MWs as compared to the glacier samples.

Some of the major bacterial orders in the glacier and MWs include *Burkholderiales, Sphingomonadales, Methylophilales Flavobacteriales, Actinomycetales,* and *Cytophagales.* In the Kongsfjorden waters, class α- proteobacteria (34.5 - 57.2%), Verrucomicrobiae (6.2 - 24.3%), γ-proteobacteria (7.6 - 16%), Flavobacteriia (9 - 17.38%), and Acidimicrobiia (1.8 - 13.4%) were found to be predominant (**Fig. 3.6b**). The dominant bacterial orders observed in the fjord waters include *Alteromonadales, Oceanospirillales, Rhodobacterales,* and *Rickettsiales* (**Fig. 3.6c**).

In the present study, 7 genera namely, *Salinibacterium, Fluviicola, Flavobacterium, Sediminibacterium, Sphingomonas, Alteromonas,* and *Pseudomonas* were found to be shared between all 3 sampling sites (VB glacier, MWs, and fjord waters). While studying the abundance of dominant OTUs (Top 25) we could observe many of the OTUs were belonging to unknown genera of the members of *Burkholderiaceae* in the glacier snow samples followed by the genus *Sphingomonas, Methylobacterium,* and *Burkholderia*. In the glacier ice, the dominance of genera *Flavobacterium, Leptolyngbya, Polaromonas, Sandarakinorhabdus,* and *Methylotenera* could be observed. OTUs belonging to the genera *Albidiferax, Flavobacterium, Leptolyngbya, Polaromonas, Methylotenera,* and *Bdellovibrio* contributed to the major bacterial fraction in MW samples. While, in the Kongsfjorden samples, the most abundant genera present were *Sulfitobacter, Pelagibacter, Octadecabacter, Marimicrobium,* and unknown genera belonging to *Verrucomicrobiaceae* along with substantial contributions from *Loktanella, Fluviicola, and Polaribacter*.

The comparison of the relative abundance of culturable bacterial species within the NGS (Next generation sequencing) dataset was also performed by blasting the sequences of bacterial isolates retrieved, against the 16S rRNA dataset of NGS created by using the blast version 2.7.1 installed within the Linux system. It was observed that except for the species *Polaromonas eurypsychrophila* which was found to be the dominant β-proteobacterial representative in the retrievable fraction from the MWs (0

- 57.14% of the total bacterial isolates in the MWs), all other species retrieved contributed to <1% abundance of the total diversity obtained by NGS. The relative abundance of *Polaromonas eurypsychrophila* was found to be 0.4 - 5.5% of the total in the MWs and 3.1% of the total in VB ice in the NGS results.

The dominance of the genus *Flavobacterium* from all the three sampling sites in both culture-based and high throughput sequencing approaches was also noted. In the culture-based study, *Flavobacterium* accounted for about 16% of the total bacterial genera retrieved from VB ice, up to 24.5% of the MW bacterial community, and up to 1.6% of the fjord bacterial community, while in the NGS based approach *Flavobacterial* genera accounted for about 17.8% of VB ice community, 4.35 - 20.9% of the total MW community and 0.35 - 1.96% of the fjord community. Similarly, the presence of genus *Salinibacterium* could be observed in both MWs as well as in the fjord system in both our study approaches. The dominance of the genus *Polaromonas* from MWs and *Loktanella* from the fjord waters was also observed from both culture based and high throughput sequencing study approaches.The Shannon diversity index, a proxy for richness and evenness was found to be highest for the MW samples (6.26 - 10.67) with the highest value noted for MW5. The snow sample VBSS1 exhibited the least Shannon index value, 1.877 while VBSS2 had an index of 5.9. The Kongsfjorden waters exhibited a Shannon index of 6.17 - 7.9, with the highest value noted for outer fjord station KNS1 (**Table 3.6**). The Chao_1 values and Observed species index values were found to be highest for the MW samples as compared to the glacier and fjord samples (**Table 3.6**).

3(a). 3.4. Differences in the bacterial community structure between the sampling sites and their correlation with environmental parameters

The Hierarchial clustering analysis based on the relative abundance of the total bacterial OTUs present within the 13 samples, yielded three clusters that corresponded to the three major sampling sites (Glacier, MWs, and fjord waters) (**Fig. 3.7**). The Heatmap plot depicted clear variation in the majority of the bacterial OTUs present within these three ecosystems (**Fig. 3.7**). On analyzing the dominant OTUs from the heat map plot, it was observed that the OTUs belonging to *Burkholderiaceae* and

Sphingomonadaceae were significantly abundant in the glacier snow samples while the OTUs belonging to *Rhodobacteraceae*, *Verrucomicrobiaceae* and γ-proteobacteria-clade OM60 dominated all 4 fjord samples.

Table 3.6.
List of obtained OTUs and associated diversity indices for each sampling site.

Sample	Total OTUs	Goods coverage	Shannon	Simpson	Chao1	Observed species
VBSS1	451	0.999	1.877	0.596	694.786	449
VBSS2	5184	0.998	5.907	0.933	5248.399	5154
VBICE	6588	0.991	8.247	0.990	9369.747	6359
MW1	7232	0.986	6.260	0.949	12172.637	7148
MW2	6505	0.993	6.655	0.957	8522.597	6408
MW3	18397	0.977	9.366	0.986	25308.411	18145
MW4	19482	0.967	10.285	0.994	26482.032	19226
MW5	19456	0.978	10.677	0.997	24137.526	19080
MW6	12187	0.991	10.516	0.997	15969.262	12031
KNBR	7381	0.986	6.933	0.959	10306.469	6795
KNS1	9880	0.989	7.915	0.979	10260.769	8847
KNS9	5358	0.988	6.176	0.952	8237.557	5014
KNS10	6145	0.989	6.274	0.954	8156.120	5575

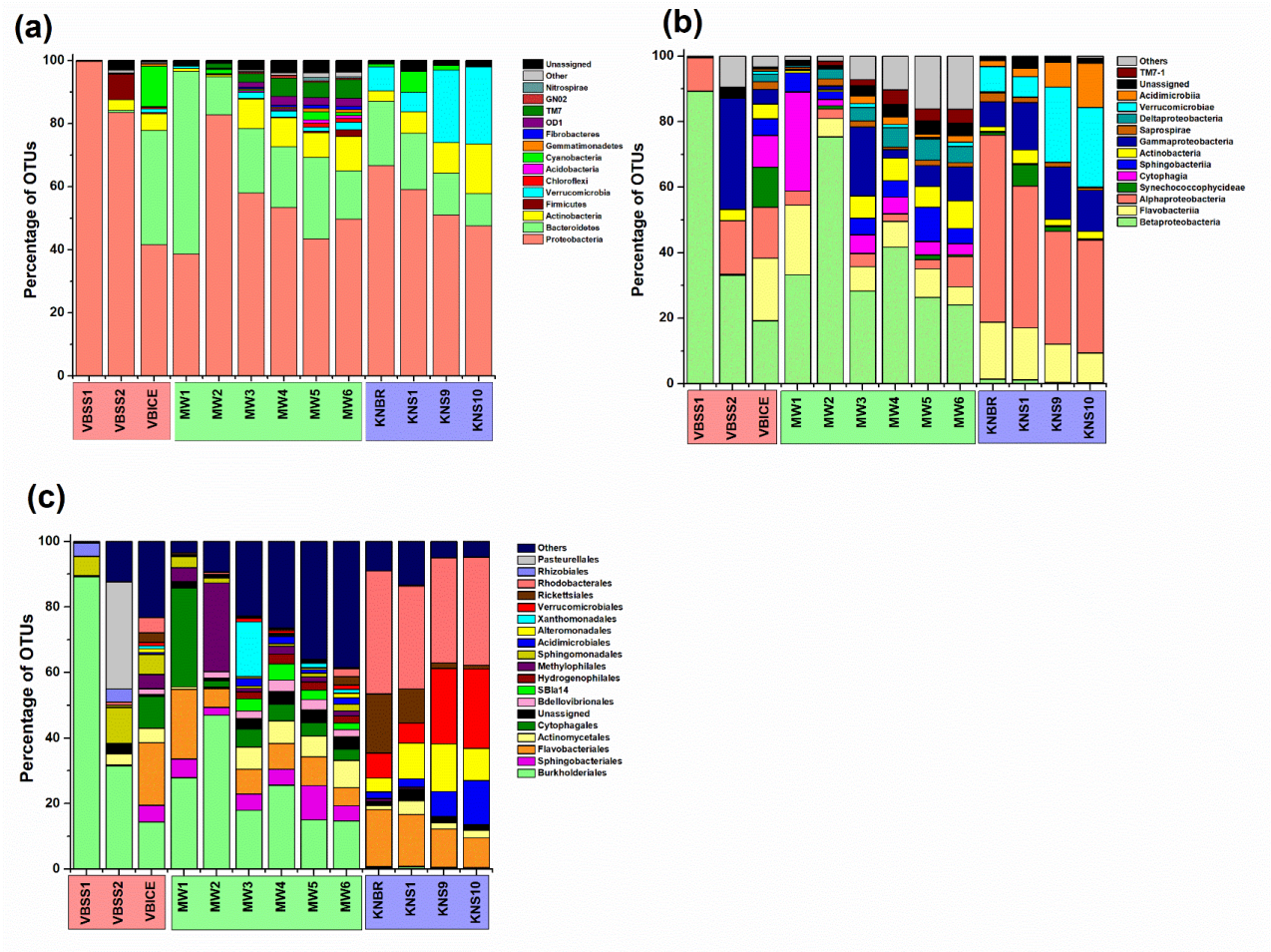

Fig. 3.6. The relative abundance of the **(a)** different phyla **(b)** different classes **(c)** different bacterial orders observed in the study. The sampling sites are marked in 3 different colors- Red - VB glacier snow and ice, Green – Meltwaters, and Blue– Kongsfjorden. The category others include all the taxa below the abundance cut off 1.5%.

Table 3.7.
Analysis of Similarity (ANOSIM) between sampling sites.

S.no	Sample Groups	R-value	Level of significance
1.	Glacier, MW, and Fjord pooled	0.873	0.001
2.	Glacier and MWs	0.722	0.012
3.	Glacier and Fjord	0.759	0.029
4.	MWs and Fjord	1	0.005

ANOSIM (testing whether two or more groups are significantly different) was calculated between all categories based on the abundance of total bacterial OTUs obtained. Each pairwise comparison of two groups was performed using 1000 permutations.

MWs exhibited the dominance of OTUs belonging to *Commamonadaceae* and *Flavobacteriaceae* which was even found to be dominant in the VB ice samples (**Fig. 3.7**). An ANOSIM test (R= 0.873, p= 0.001: Bray-Curtis) also supported that the three sampling sites harbored significantly different bacterial communities **(Table 3.7)**. The Venn diagram demonstrated the number of site-specific OTUs and also the common OTUs shared between the sites (**Fig. 3.8)**. The Venn diagrams were plotted taking into consideration all the OTUs present at least 1 station within each sampling site (**Fig. 3.8a)** and also the OTUs that are present at all the stations within each sampling site (**Fig. 3.8b**) and **Fig. 3.8c)**. The Venn diagram with only the VBice component along with the shared OTUs of MWs and fjord (shared OTUs - the OTUs that are present at all the stations within each sampling site) demonstrated that 13% of VB ice OTUs were shared with all the MW stations while 8.6% of OTUs were shared with all the fjord stations **(Fig. 3.8c).** It was also observed from **Fig. 3.8c** that MWs shared 75% of their total shared OTUs with the VB ice while only 35% of the shared OTUs of the fjord was common to that of VB ice.

The overall average dissimilarity between the VB glacier, MWs and the Kongsfjorden samples using SIMPER analysis was 95.23%. The OTU 1105280 (9.15%) and OTU 331 (6.5% belong to *Burkholderiaceae* and the OTU 101660 (5.76%) and OTU 520 (5.07%) belonging to *Rhodobacteraceae* contributed the maximum to the overall dissimilarity between the samples (**Table 3.8**). The OTU 1105280 (*Burkholderiaceae*)

contributed the maximum to the significant difference between the glacier and meltwater (16.85%) as well as between glacier and marine fjord water (15.89%) while the OTU 101660 contributed maximum to the significant differences between MW and fjord water (9.2%).

The Bray-Curtis distance-based RDA (db-RDA) plot revealed the clustering of sampling sites by the relative abundance of different bacterial classes and various environmental parameters (temperature, nutrients, anions, and trace metals) (**Fig. 3.9**). The close association of the nutrients (NO_3^-, SiO_4^{2-}) with the glacier ice and MW bacterial community and that of temperature, trace metals (Mn, Fe, Ni, Cu, and Zn), Cl^-, and SO_4^{2-} ions with the fjord community could be observed from the db-RDA plot.

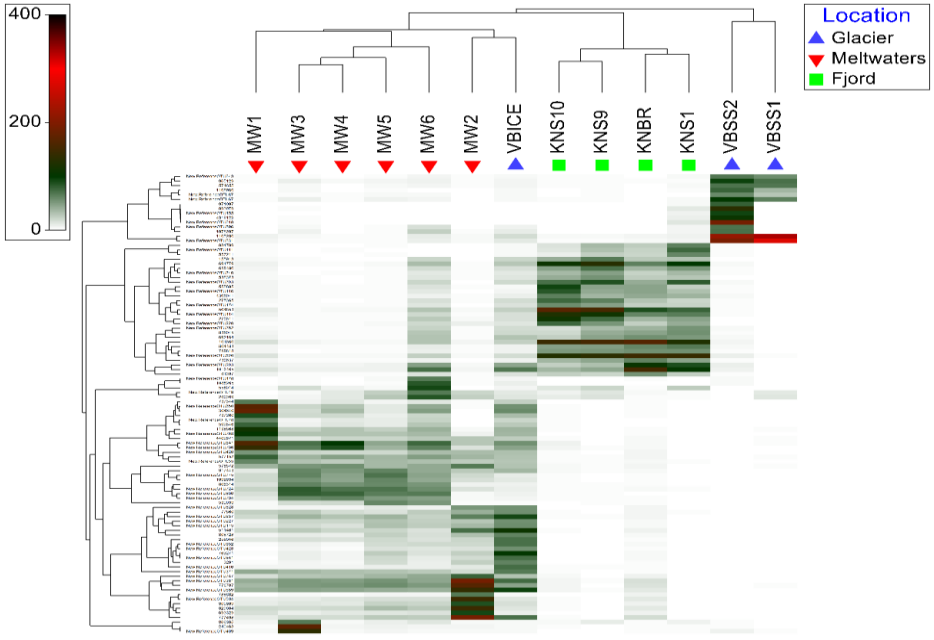

Fig.3.7. Double hierarchial dendrogram showing the bacterial distribution among the thirteen samples. The cluster dendrogram was standardized by square-root transformation and a Bray–Curtis dissimilarity matrix was generated. The heatmap plot depicts the relative abundance of the top 100 bacterial OTUs (variables clustering on the Y-axis) within each sample (X-axis clustering). The relative values for the bacterial OTU abundance are shown by the color intensity indicated at the top left corner of the figure respectively.

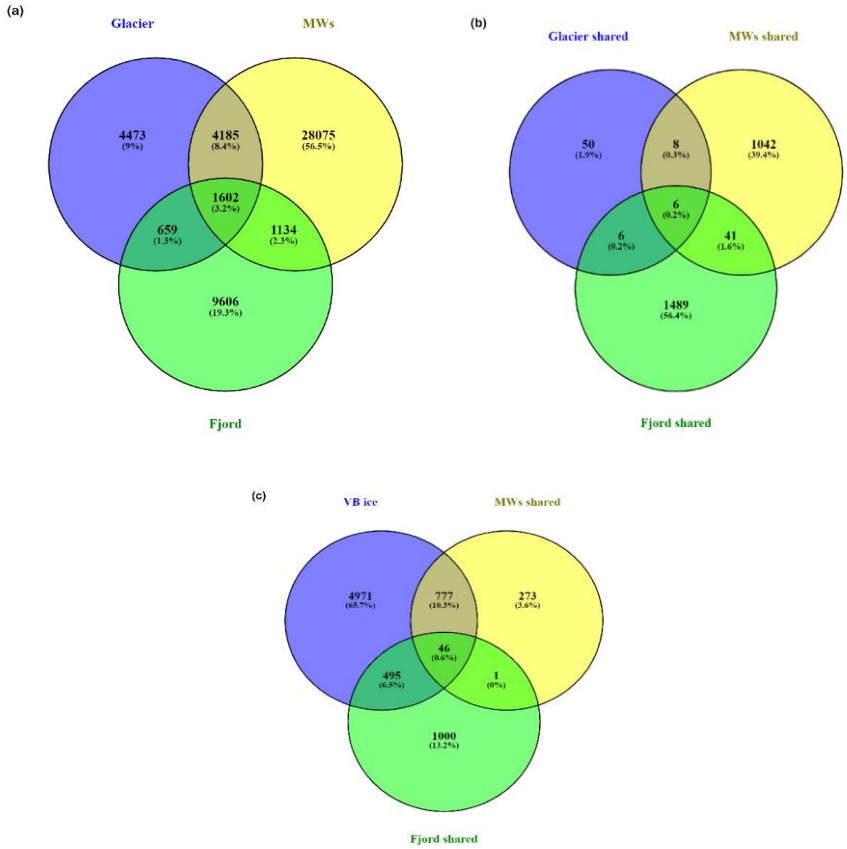

Fig. 3.8 (a) Venn diagram displaying the degree of overlap of bacterial OTUs among the three sampling sites taking into consideration all the OTUs present in at least 1 of all the stations within each group, **(b)** Venn diagram indicating the degree of overlap of bacterial OTUs among the three sampling sites taking into consideration of only the shared OTUs present in each sampling group (shared OTUs means OTUs that are present in all the stations within each group), **(c)** Venn diagram indicating the degree of overlap of bacterial OTUs among the VB ice, shared OTUs of MWs and shared OTUs of Fjord waters.

Table 3.8.
Average dissimilarity contribution of the quantitatively prominent OTUs in the VB glacier, MWs and Kongsfjorden after SIMPER.

OTU	Phylum	Class	Order	Family	Average dissimilarity (%)			
					All pooled	Glacier & MW	MW & Fjord	Glacier & fjord
OTU 1105280	*Proteobacteria*	*β-Proteobacteria*	*Burkholderiales*	*Burkholderiaceae*	9.152	16.85	0.01051	15.89
OTU 331	*Proteobacteria*	*β-Proteobacteria*	*Burkholderiales*	*Burkholderiaceae*	6.531	12.02	0.00725	11.34
OTU 101660	*Proteobacteria*	*α-Proteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	5.764	0.1192	9.207	7.345
OTU 520	*Proteobacteria*	*α-Proteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	5.071	0.06987	8.124	6.467
OTU 564853	*Verrucomicrobia*	*Verrucomicrobiae*	*Verrucomicrobiales*	*Verrucomicrobiaceae*	4.251	0.1169	6.763	5.429
OTU 691779	*Proteobacteria*	*γ-Proteobacteria*	*Alteromonadales*	*OM60*	2.772	0.1511	4.388	3.469
OTU 1016465	*Proteobacteria*	*α-Proteobacteria*	*Rickettsiales*	*Pelagibacteraceae*	2.689	0.8042	3.951	2.994
OTU 641	*Proteobacteria*	*β-Proteobacteria*	*Burkholderiales*	*Comamonadaceae*	2.446	2.583	3.347	0.4363
OTU 414	*Verrucomicrobia*	*Verrucomicrobiae*	*Verrucomicrobiales*	*Verrucomicrobiaceae*	2.274	0.04862	3.627	2.907
OTU 381	*Proteobacteria*	*β-Proteobacteria*	*Burkholderiales*	*Comamonadaceae*	2.162	2.4	2.673	0.7821

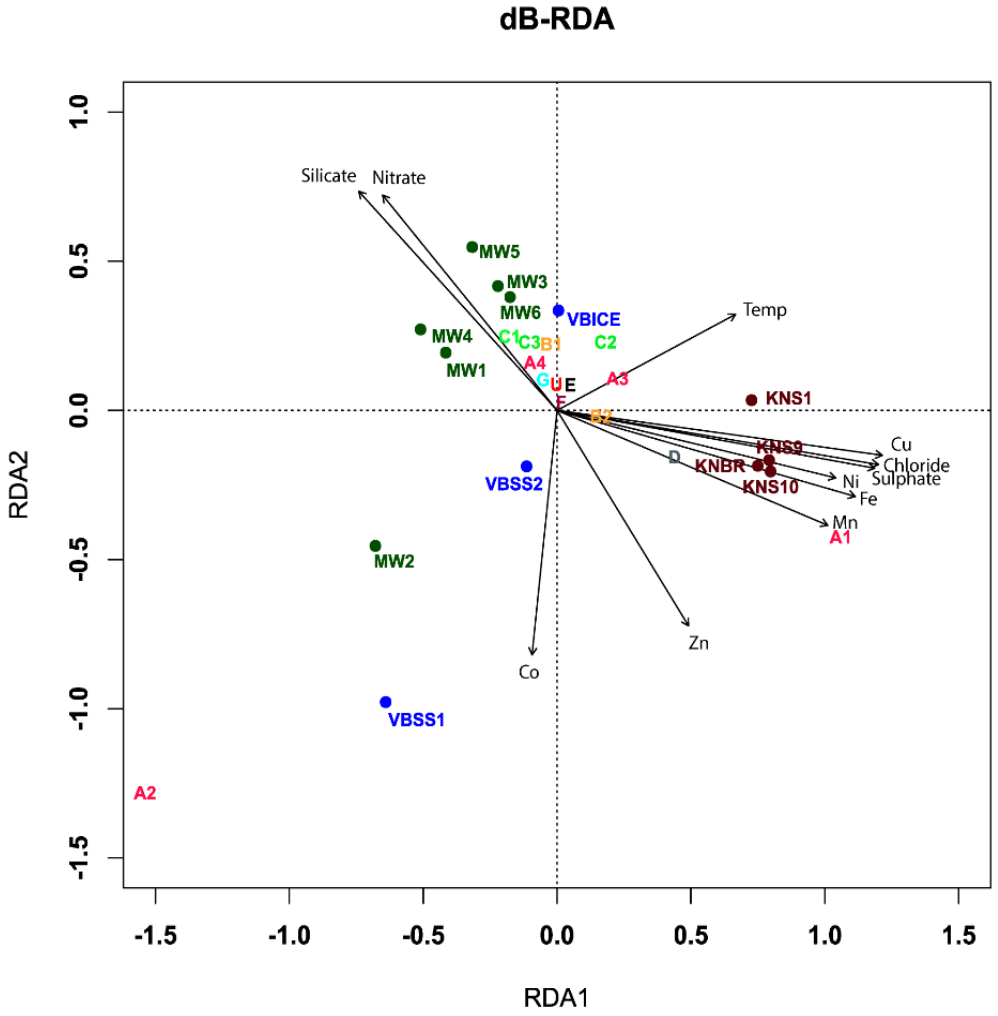

Fig. 3.9. Distance-based redundancy analysis to show correlations between the bacterial communities and environmental factors of the 13 samples from the three sampling sites. The arrows represent environmental factors measured such as nutrients, anions and trace elements. The 13 samples are labelled with a unique sampling code and dots in blue for VB glacier snow and ice, dark green for MWs, and dark brown for Kongsfjorden waters. The length of the arrows indicates the relative importance of that environmental factor in explaining the variation in the bacterial communities, while the angle between each arrow and the nearest axis indicates the closeness of the relationship between each other. The bacterial classes are represented in different colors with unique alphabetical codes. The bacterial classes: A1 - α-Proteobacteria, A2 - β-Proteobacteria, A3 - γ-Proteobacteria and A4 - δ-Proteobacteria, B1 - Actinobacteria and B2 – Acidimicrobiia, C1 - Cytophagia, C2 - Flavobacteriia, and C3 - Sphingobacteriia, D - Verrucomicrobiae, E - Synechococcophycidiae, F - Saprospirae, G - TM7-1, U - Unassigned. The environmental parameters include Temp-temperature, Nitrate, Silicate, Chloride, Sulphate, and trace elements such as Mn-Manganese, Fe-Iron, Ni-Nickel, Co-Cobalt, Cu-Copper, and Zn-Zinc.

3(a). 4. Discussion

In the present study, we compared the bacterial diversity of Vestrebroggerbreen valley glacier, its associated pro-glacial channels, and the downstream marine ecosystem of Kongsfjorden using culture-dependent and culture-independent amplicon-based high throughput sequencing techniques, to understand the degree to which the transport of microbes from the glacier system impose an effect on the patterns of diversity in the downstream ecosystem.

The dominance of sequences affiliated with β-Proteobacteria in the VB glacier snow (89.3% in VBSS1 and 33.2% in VBSS2) corresponds to the studies conducted previously in the Arctic from glacier ice (Lutz et al., 2017) and glacier snowpacks (Hell et al., 2013; Larose et al., 2010). Due to their rapid response to environmental fluctuations, β-Proteobacterial class is characterized as the r-strategists in aquatic as well as in terrestrial systems and are known for exploiting spatially and temporally variable resources which explain their potential to proliferate in transient niches such as the glacial snow (Hell et al., 2013). Among β-Proteobacteria, the sequences affiliated to the order *Burkholderiales* with family *Burkholderiaceae* were present in higher abundance in the snow samples. The majority of the members belonging to this order are metabolically and ecologically diverse, known for their ability to fix atmospheric nitrogen and utilize a wide range of organic compounds as carbon sources (Coenye and Vandamme, 2003). *Burkholderiales* have often been found to be dominant in sediments beneath glaciers and subglacial ecosystems (Skidmore *et al.*, 2005) and are known to use organic matter eroded from glaciers and transported by glacial meltwater (Algora et al., 2015). The comparatively higher Mn and Fe values in the snow (Table 2) could also support the abundance of *Burkholderiales in the glacier environment* as they are known for the reduction of Mn and Fe under aerobic conditions (Algora et al., 2015). Thus, their presence in the glacier snow is an indication of their significant contribution to nutrient cycling within the glacier ecosystems. The dominance of sequences belonging to *Sphingomonas* in the top and middle layers of snow have been reported by Møller, et al. (2013) while the dominance of *Methylobacterium* spp. from ice core samples from Antarctica along with their ability to metabolize single carbon substrates

to cope with the oligotrophic conditions in the ice has been reported by Antony et al. (2012). The low nutrient concentrations observed in the snow samples (Table 1) could explain the abundance of these genera which are capable of surviving such cold oligotrophic conditions.

The accumulation zone snow (VBSS1) community was majorly driven by the β-proteobacterial class while an increased presence of heterotrophic γ-proteobacterial class was noted in the ablation snow sample VBSS2 (33.9%). This could be possibly due to the deposition from terrestrial dust or aerosols (aeolian) or could be associated with the presence of supraglacial features such as cryoconites which provide dissolved nutrients and organic carbon, favoring the abundance of the heterotrophic bacterial community (Xiang et al., 2009). The highly diverse VB glacier ice community dominated by members belonging to Proteobacteria and Bacteroidetes can be linked to the higher nutrient concentrations observed in the glacier ice, compared to the snow samples (**Table 1**) which in turn can be due to the deposition of sediments, organic and inorganic debris on the snout ice through aeolian as well as post-depositional events (Xiang et al., 2009). The higher abundance of OTUs belonging to Synechococcophycideae (Cyanobacteria) in the VB glacier ice can be related to the light rich conditions in the glacier surface and suggests that this bacterial group could be one of the common phototrophs in the glacier system sustaining the dynamics of the heterotrophic bacterial community in the glacier and pro-glacier environment (Xiang et al., 2009).

The MW bacterial community was comparable to the glacier ice bacterial community wherein the major players were β and γ -proteobacteria along with significant contributions from the phyla Bacteroidetes (classes Cytophagia, Flavobacteriia, and Sphingobacteriia). The presence of many candidate phyla exclusively in the MW samples is an indication that the pro-glacial channels although being transient niches support diverse and rare bacterial communities. The bacterial genera that were repeatedly encountered in the NGS analysis of MWs included *Flavobacterium, Leptolyngbya, Polaromonas,* and *Methylotenera* many of which could be traced back to the VB glacier snout ice community. The OTUs affiliated with the genus *Polaromonas* are known to oxidize a wide variety of substrates in cold environments

and recent genomic studies have indicated their alternative dormancy mechanism as a survival strategy to colonize extreme glacial environments and recently deglaciated areas in higher abundance (Darcy et al., 2011). The *Flavobacterium* affiliated OTUs were previously noted from low salinity, cold environments such as polar lakes, streams, rivers, and cold muddy soils (Bernardet and Bowman, 2006) while the cyanobacterial genus *Leptolynbya* are known for their significant ecological role in various microbiocenoses in coastal deglaciated zones (Komárek, 2007) and their photosynthetic activity could be critical in governing the glacier dynamics. Likewise, the presence of methylotrophic bacteria belonging to the genera *Methylotenera* from the MWs is an indication of the functional versatility of the bacteria associated with the transient MW ecosystem. The MW channels acting as a conduit between the glacier and the downstream fjord systems are subjected to daily and seasonal variability which includes changes in the temperature, sediment load, stream discharge volume, nutrient flux, and redox conditions (Hotaling et al., 2017). Also, the time that the bacteria spend in the MW channels (residence time) is too short to establish a stable bacterial community in such niches (Crump et al., 2007). This could explain the high diversity and functional plasticity of the resident bacterial community in the MW channels. A higher fraction of unknown bacterial genera observed in MWs suggests the potential of this unexplored ecosystem which might be crucial in understanding the MW channel dynamics in the light of enhanced glacial retreat.

The predominance of sequences affiliated to α-Proteobacteria, Flavobacteriia, Verrucomicrobia, γ-Proteobacteria, and Acidimicrobiia in the Kongsfjorden waters is as per the previous reports from Kongsfjorden (Zeng et al., 2013; Sinha et al., 2017b; Jain and Krishnan 2017b). The relatively higher abundance of OTUs belonging to the genera *Sulfitobacter, Octadecabacter,* and *Loktanella* (order *Rhodobacterales*, α-Proteobacteria) could be observed in the fjord water samples. Bacteria belonging to the genera *Rhodobacterales* are widespread and abundant in marine environments and their distribution and diversity in a Canadian Arctic marine system were elaborately evaluated by Fu et al. (2013). The most common *Roseobacter* clade belonging to *Sulfitobacter* and OTUs belonging to *Octadecabacter* as the primary surface colonizers and their role in community succession in the temperate coastal waters was reported

by Dang et al. (2008). Members of *Sulfitobacter* and *Loktanella* were reported to be the predominant part of particle-associated bacterial fraction in Kongsfjorden waters (Jain and Krishnan, 2017b) and are known to be associated with organic and inorganic marine particles (Slightom and Buchan, 2009). Similarly, the dominance of OTUs belonging to *Pelagibacter ubique* belonging to the SAR11 clade of α-Proteobacteria in the outer KNS1 and KNBR stations is consistent with the results obtained by Sinha et al. (2017b). The ultramicrobial structure of *Pelagibacter sp.* helps them to escape from the protistan grazing and makes them more competitive (Zhao et al., 2017), and their tolerance to a wide range of salinity and temperature makes them an abundant class in marine habitats (Sinha et al., 2017b). The presence of the genera *Marimicrobium* belonging to the OM60 clade (γ-Proteobacteria) is observed within the top 10 OTUs in all the 4 fjord water samples and this clade is reported to be a marine representative (Yan et al., 2009). Similarly, the presence of OTUs belonging to the class Flavobacteriia was observed among the top 25 OTUs from the fjord stations (*Polaribacter, Fluviicola, and uncultured Flavobacteriaceae*), the presence of which can be linked to their significant role in the biogeochemical cycling of nutrients in the marine waters (Sinha et al., 2017b). Jain and Krishnan (2017b) have suggested the role of Flavobacterial members such as *Polaribacter* in the degradation of phytoplankton-derived dissolved substrates. The relatively higher abundance of OTUs affiliated to Verrucomicrobia observed in the inner fjord stations as compared to the outer station can be linked to the higher polysaccharide biomass associated with the inner fjord due to the brown algae/kelp ecosystem distributed along the coastal and inner zones (Buchholz and Wiencke 2016). The relatively higher abundance of OTUs belonging to the genera *Synechococcus* (Cyanobacteria) in the outer Kongsfjorden station as compared to the inner fjord stations, is an indication for the increased Atlantic water intrusion into the Kongsfjorden (Sinha et al., 2017b, Paulsen et al., 2016). The higher total organic carbon values obtained from the Kongsfjorden waters (21.7 - 31.1ppm) indicate the enhanced organic matter inputs in Kongsfjorden which in turn could be supporting evidence for the dominance of heterotrophic bacterial community known for complex organic matter degradation in the fjord. The dominant bacterioplankton community can have a great influence on the environment that they inhabit by

contributing greatly to organic compounds cycling, such as the carbon cycle and it is been reported that approximately, 50% of the organic carbon is produced through heterotrophic bacterial metabolism (Ducklow 2000; Bunse and Pinhassi, 2017). In the present study, the most dominant OTUs obtained belonged to genera *Octadecabacter*, *Pelagibacter*, OTUs belonging to *Flavobacteriaceae, Alteromonadaceae*, etc which possess the ability to metabolize carbon. Thus, the overall bacterial community structure in the Kongsfjorden waters provided some insights into the functional role of fjord bacteria in organic matter degradation which is critical for keeping the ocean ecosystem functioning at a healthy ratio.

Although next-generation sequencing technologies have opened a new dimension in unravelling the rare biosphere, most of the abundant taxa obtained from the metagenomic data are difficult to grow in the laboratory conditions which could limit our understanding of their physiological and functional potential in depth. Hence, culture-based techniques stand significant in isolation and identification of bacterial strains and characterization of their physiology which can be correlated with the culture-independent methods.

Diverse culturable bacteria were obtained from the glacier ice and MW samples and most of the isolated strains exhibited pigmentation which could be a possible UV protectant mechanism for their survival in the glacier environment (Lutz et al., 2017). It was observed from our study that dilute TSA media ($1/10^{th}$ strength) yielded the maximum CFU count in the first cultivation for the glacier ice and MW samples (**Table 3.5**), which is in accordance with the previous works done by Antony et al. (2012) wherein they obtained higher retrieval yield on dilute TSA medium (1 and 10%) for the Antarctic ice core samples. The fact that oligotrophic bacteria cannot grow at higher substrate concentrations and cannot grow in nutrient-rich media upon first cultivation of organisms from natural habitats (Fry, 1990; Cho and Giovannoni, 2004) well explains our results. The inability to retrieve bacteria from the VB snow samples could be associated with their very low nutrient values as compared to the glacier ice, MWs, and fjord waters **(Table 3.1)**. For example, the nitrite values in VB snow were 15 times lower as compared to VB ice and 4 times lower as compared to the MWs while the

nitrate values were 16 times lower than ice and about 70 times lower than MWs. A similar trend was observed for phosphate and silicate values of snow also.

We could observe that among the VB ice isolates, except for the species *Paucibacter toxinivorans*, all others were previously reported from the freshwater ecosystem, of which 50% of the isolates are reported from the cold environment of glaciers. Similarly, about 46% of the strains isolated from the MWs have been previously reported from the cold habitats such as glacier ice, permafrost, tundra ecosystem, microbial mats, temporary ponds, sediments, and glacier cryoconites (**Table 3.4**) which testifies their adaptation to cold conditions. Among the VB ice isolates, *Polymorphobacter glacialis* was found to be the dominant species followed by species representing *Flavobacterium, Herminiimonas,* and *Cryobacterium.* The first report of *Polymorphobacter glacialis* is from the ice core of Muztag glacier, China (**Table 3.4**). The dominance of genus *Flavobacterium* from both Arctic and Antarctic ice packs by cultivation was reported Brinkmeyer et al. (2003) while there are reports on the presence of *Herminiimonas* sp. from the Antarctic glacier (García-Echauri et al., 2011) and Greenland sea-ice brine (Møller et al., 2011). The Actinobacterial genera *Cryobacterium* have been reported as one of the dominant bacterial taxa from the cryoconite hole samples from Broggerbreen glaciers, from Ny-Ålesund (Singh et al., 2014) and were reported for their cold-active enzyme activities. The majority of the isolated strains from the MWs belonged to *Pseudomonas* sp. which are previously reported from the Arctic region and known for their cold-active enzyme production (Reddy et al., 2009). Production of cold-active enzymes can help the bacteria to thrive under glacier-cold conditions and to utilize the available organic matter in the oligotrophic environments of the glacier and proglacial channels (Singh et al., 2016). The isolates belonging to the order *Burkholderiales* were previously reported for their metabolic diversity and niche-specific functions such as cyanobacterial hepatotoxin degradation, hydrogen oxidation, and pigment production (**Table 3.4**). However, the isolates obtained in this study have not been tested for this phenotype. Few bacterial genera like *Polaromonas, Sphingomonas,* and *Flavobacterium* which were identified through an amplicon sequencing-based approach were also observed to be a dominant fraction in the culture-based study. Among the bacterial isolates retrieved from the

meltwaters, a minor fraction was observed to be closely affiliated to bacteria previously reported from Arctic tundra (*Pseudarthrobacter oxydans*) tolerant to toxic metal mercury (Balan et al., 2018) and from the Tibetan permafrost (*Pseudarthrobacter sulfonivorans)* known for degradation of crude oil and multi benzene compounds (Zhang et al., 2016) (**Table 3.4**). These observations suggest the highly flexible nature of the MW isolates to withstand various environmental stresses and hence a detailed genome-based study on such candidates is a prerequisite to understanding how they have evolved such resistant mechanisms in the cold Polar environment. The cultivable fraction of bacterial communities from the glacier ice and MWs also revealed the presence of potential novel bacterial isolates (with <98.7% similarity with closest phylogenetic neighbors) which is by the previous studies that showed the presence of rare taxa in glacially fed streams (Wilhelm et al., 2014). These rare communities might be lost with the ongoing retreat of glacial snow and ice driven by climate change, therefore systematic studies of such glacial ecosystems are critical.

For the Kongfjorden water samples, maximum retrievable counts were obtained from Zobell Marine Agar ($1/4^{th}$ strength) **(Table 3.5)**. Previous studies have reported the use of ZMA media for the enumeration of viable heterotrophic bacteria from the marine environment of Kongsfjorden (Sinha et al., 2017a; Prasad et al., 2014; Srinivas et al., 2009). Among the isolates retrieved from Kongsfjorden waters, 63% were previously reported to be of marine origin and 31% were of freshwater origin and about 56% of the total were found to be isolated from cold environments/Polar regions. (**Table 3.4**). The γ-proteobacterial members belonging to the genera *Alteromonas, Pseudoalteromonas, Pseudomonas,* and *Psychrobacter* retrieved in our study were previously reported for their extracellular enzyme production and role in the degradation of dissolved organic matter and carbohydrates in the cold marine environment of Kongsfjorden (Jain and Krishnan, 2017a). Among the α-proteobacterial candidates, the dominance of phylotype affiliated with *Loktanella salsilacus* could be observed which were previously reported from Kongsfjorden waters (Sinha et al., 2017b; Jain and Krishnan 2017b).

While comparing the VB ice, MWs, and fjord system, it was observed that the majority of the culturable isolates identified from VB ice and MWs were retrieved from 4 °C

incubations while the isolates identified from Kongsfjorden waters were more from 20 °C. This could explain the differences in the retrievable bacterial community between the systems, wherein the glacier-associated system with its cold temperatures supports more of the cold-loving bacterial community while the fjord system with comparatively warmer waters supports bacteria that could grow better at higher temperature incubations (20 °C).

The detection of isolates/OTUs belonging to the genus *Flavobacterium* from all three sampling sites in both culture based and high throughput sequencing approach strongly indicates the adaptability of this genus to cope with salinity and temperature fluctuations associated with the freshwater system of glacier and MWs as well as the marine fjord system. Similar conclusions can be drawn for the presence of *Salinibacterium* sp. in both MWs as well as fjord system. This might also suggest that such species have the potential to thrive in oligotrophic conditions which prevail in the glaciers and MWs as well as the high organic-rich conditions that exist in the fjord waters. Comparison of the culturable data with the high-throughput sequencing data revealed that out of the 42 genera retrieved in the cultivable approach, 25 were detected in amplicon sequencing-based approach, while 17 genera were represented only by the culturable isolates (**Table 3.4**). Similar observations were reported by Chen et al. (2016) and Sinha et al. (2019). In the former paper, the authors while comparing the actinobacterial genera using culture-dependent and pyrosequencing approach observed that 14 actinobacterial genera in the cultivable approach were not detected in the pyrosequencing data. In the latter paper, the authors while comparing the bacterial community structure using cultivation-dependent and cultivation-independent (16S rRNA amplicon sequencing) approach, reported the retrieval of 5 bacterial genera in the cultivable approach which was not detected in the high throughput sequencing data. These results reveal the importance of the culture-based approach in bacterial diversity studies associated with the rare biosphere of the Arctic. The potential novel bacterial isolates obtained in our culture-based study also suggest the significance of culturing bacteria as a tool to unravel the unknown taxa and for understanding their functional roles in the ecosystem. Therefore a combination of NGS-based and culture-based approaches is a prerequisite in obtaining a comprehensive picture of bacterial diversity**.**

The glacier snow, MWs, and fjord waters exhibited significant differences in their bacterial community composition as per the cluster dendrogram and heatmap plot **(Fig. 3.7).** The top abundant OTUs that were repeatedly encountered in the NGS analysis of MW samples were also found to be dominant in the VB ice. The Venn diagrams plotted could also explain the same in detail. From **Fig. 3.8c**, it is evident that VB ice shared more number of OTUs with the MWs as compared to the fjord waters. The comparable nutrients, ions, total organic carbon, and trace element values for glacier ice and MWs also suggest the similar nature of these systems which supports the presence and abundance of the same bacterial OTUs. Since environmental conditions are known to shape bacterial community compositions, the presence of shared OTUs in similar environmental conditions may not be the only reason that can conclusively indicate sourcing. Further quantitative analyses of the data in this regard is expected to give better clarity on the same.

A significant contribution of the OTU 1105280 and OTU 331 belonging to *Burkholderiaceae* and the OTU 101660 and OTU 520 belonging to *Rhodobacteraceae* to the overall % dissimilarity between the samples was observed in the SIMPER analysis. The high proportion of sequences belonging to *Rhodobacteraceae* in the fjord samples could be linked with their particle-associated nature enabling them to associate with organic and inorganic marine particles/surfaces in the Kongsfjorden (Jain and Krishnan, 2017b) while the dominance of OTUs belonging to *Burkholderiaceae* in the VB glacier snow highlights their resistance to cold oligotrophic conditions of glaciers and free-living nature (Coenye and Vandamme, 2003).

Analysis of linkages between the bacterial community structure and the various significant environmental parameters using distance-based RDA yielded a strong correlation of nutrients (NO_3^- and SiO_4^{2-}) with glacier ice as well as the MW bacterial community. Glaciers in Svalbard, receive modest rates of nutrient deposition that may be highly variable from year to year due to extreme episodic events (Hodson et al., 2005). The supraglacial environment promotes ammonium ion assimilation and nitrification within the cryoconite holes while the subglacial environment appears to promote denitrification due to the prevalent anoxic conditions (Hodson et al., 2005). The geochemical data from the Austrebroggerbreen glacier sites suggests that the

Broggerbreen glacier forefields are geochemically significant in the context of silicate weathering (Hodson et al., 2005). This could very well explain the comparatively higher nutrient values in the glacier ice and MW channels as compared to the fjord waters, which in turn might have influenced the distinct bacterial community structuring of these ecosystems. The lower nutrient values in fjord waters were comparable with the previously reported values of nutrients from the Kongsfjorden waters (Bazzano et al., 2014) and indicated photosynthetic activities to be the major cause for nutrient depletion during summer. Temperature exhibited a positive correlation with Kongsfjorden waters, which can be related to the Atlantic water intrusion to the fjord which in turn is a crucial factor determining the fjord properties (Sinha et al., 2017a). Trace metals, Cl^-, and SO_4^{2-} ions were found to be linked with the fjord bacterial community as per the db-RDA plot (**Fig. 3.9**). The bacterial community in the Kongsfjorden are mostly marine in origin, capable of tolerating high salt concentration. Anions such as Cl^-, and SO_4^{2-} ions are essential for marine bacteria to cope with the salt stress by acting as osmolytes as well as essential for their growth (Roeβler et al., 2003) while trace elements such as Fe, Mn, Zn, Cu, and Ni play a significant role in microbial cycling of major nutrients like C and N in oceans (Morel and Price, 2003). Sinha et al. (2017a) have suggested that the phytoplankton composition and concentration along with the mild temperatures in the fjord are the major determining factors for the Kongsfjorden bacterial diversity. Thus, it can be inferred that the Kongsfjorden bacterial community varies with changes in the physicochemical properties of the fjord which in turn is majorly influenced by the inflow of warm Atlantic water and is significantly different from the valley glacier and MW bacterial community.

Thus, the gradual loss in the glacier associated taxa on reaching the fjord system along with the significant variation in the bacterial community composition of the VB glacier, its associated MW channels, and the downstream marine waters of Kongsfjorden ($R = 0.873$, $p = 0.001$) suggests the influence of environmental variables and dispersal vectors in determining the patterns of bacterial diversity. However, further quantitative analyses are needed for a detailed understanding of the sourcing of bacterial communities to the downstream ecosystem from the glaciers.

3(b). Understanding the bacterial diversity and their metabolic potentials associated with the glacio-marine sedimentary environments of Arctic

3(b). 1. Introduction

Sedimentary environments represent one of the most complex microbial habitats supporting diverse bacterial communities with distinct metabolic potentials, representing the key players in organic matter remineralization (Lin et al., 2017; Jorgensen et al., 2012; Stibal et al., 2012; Teske et al., 2011). So far, there is only a single culture-dependent study reported from the sediments of a meltwater stream from Midtre-Lovénbreen glacier in Ny-Ålesund, Svalbard (Reddy et al., 2009) indicating the need for a detailed investigation on the relatively unexplored glacier snout and forefield sediments in the region and how their metabolic signature reflects the glacier environment. Similarly, studies by Zeng et al. (2017), Conte et al. (2018), Fang et al. (2019), Teske et al. (2011), and Buongiorno et al. (2019) (**detailed in 3.1**) suggest the role of environmental factors in structuring the fjord sediment community and their distinctive metabolic functions as influenced by the substrate availability in the fjord sedimentary environment. Therefore, understanding the bacterial community structure along with assessing their metabolic potentials is crucial for determining their ecological role in such complex environments.

Furthermore, in the light of enhanced warming scenario in the Arctic, with observed and projected annual average Arctic warming approximately twice the global mean (Overland et al., 2019), it is also imperative to understand the influence of temperature on the metabolic response of the total community as well as on different bacterial species of the terrestrial and fjord system. Kritzberg et al. (2010) studied the bacterial response in terms of production and respiration from the Fram strait waters by experimental manipulations of temperature and resources in combinations. They reported enhanced bacterial production and respiration following a temperature increase of +6 °C from the in-situ conditions and the response to temperature was

higher in resource amended treatments, suggestive of a substrate-temperature interaction regulating the bacterial metabolism.

Keeping these aspects in mind, we tried to address the following questions in the present study:

(a) whether the bacterial diversity associated with glacio-marine sediments is influenced by the environmental factors?
(b) whether the total sediment community metabolic response towards ecologically important substrates in the terrestrial and fjord systems is different from the metabolic response exhibited by individual bacteria isolated from such systems?
(c) whether phylogenetically-related species tend to exhibit similar metabolic responses or whether sediment-type and origin influence their metabolic responses?
(d) whether temperature variations have an influence on the metabolic profiles of the total community as well as on different bacterial species of the terrestrial and fjord system?

For the study, cultivation-dependent and cultivation-independent high throughput amplicon sequencing approach was followed accompanied with measurements of various environmental parameters associated with the glacier and fjord sediments. Community-level physiological profiling (CLPP) of the sediments using EcoPlates^{TM} and growth response of retrievable bacteria towards different carbon substrates, with varying temperatures were studied. Such studies would help in estimating and eventually predicting the bacterial responses to varying substrate availability in the highly changeable Arctic ecosystems (Thomas et al., 2021).

3(b). 2. Materials and Methods

3(b). 2.1. Study area and sampling strategy

The study area covered Vestrebroggerbreen (VB) glacier and the downstream fjord system - Kongsfjorden (**Fig. 3.10**). A total of 3 sediment samples were collected from the Vestrebroggerbreen glacier snout region (VB1 to VB3) and pooled together to form a single representative sample (VB) (**Fig. 3.11 (a))**. Similarly, 3 sediment samples were collected from the glacier foreland region (BR1 to BR3), and a homogenous single sample was obtained as BR (**Fig. 3.10** and **3.11(b)**). Surface sediment samples were collected across Kongsfjorden from the point where the meltwater channels empty into the fjord (KNBR), from the outer fjord mouth (KNS1), and the inner fjord (KNS9) region (**Fig. 3.10**) using Van-veen grab deployed from *MS Teisten*. VB and BR sediments (**Fig. 3.11 (c), (d),** and **(e)**) are representatives of the Arctic terrestrial system while KNBR, KNS1, and KNS9 sediments represent the fjord system. The overlying water temperature and salinity were measured using a Waterproof Portable meter (Cyberscan series 600) (Eutech instruments, Thermo Fisher Scientific, USA) for all the sampling locations. All the samples were collected in sterile sampling bags (Nasco Whirlpaks, Himedia, India) and were immediately transferred to -20 ˚C until further processing. An aliquot of the samples was maintained at 4°C for cultivation-dependent analysis. The samples were then transported in a frozen state to the home laboratory at National Centre for Polar and Ocean Research (NCPOR), India for analysis.

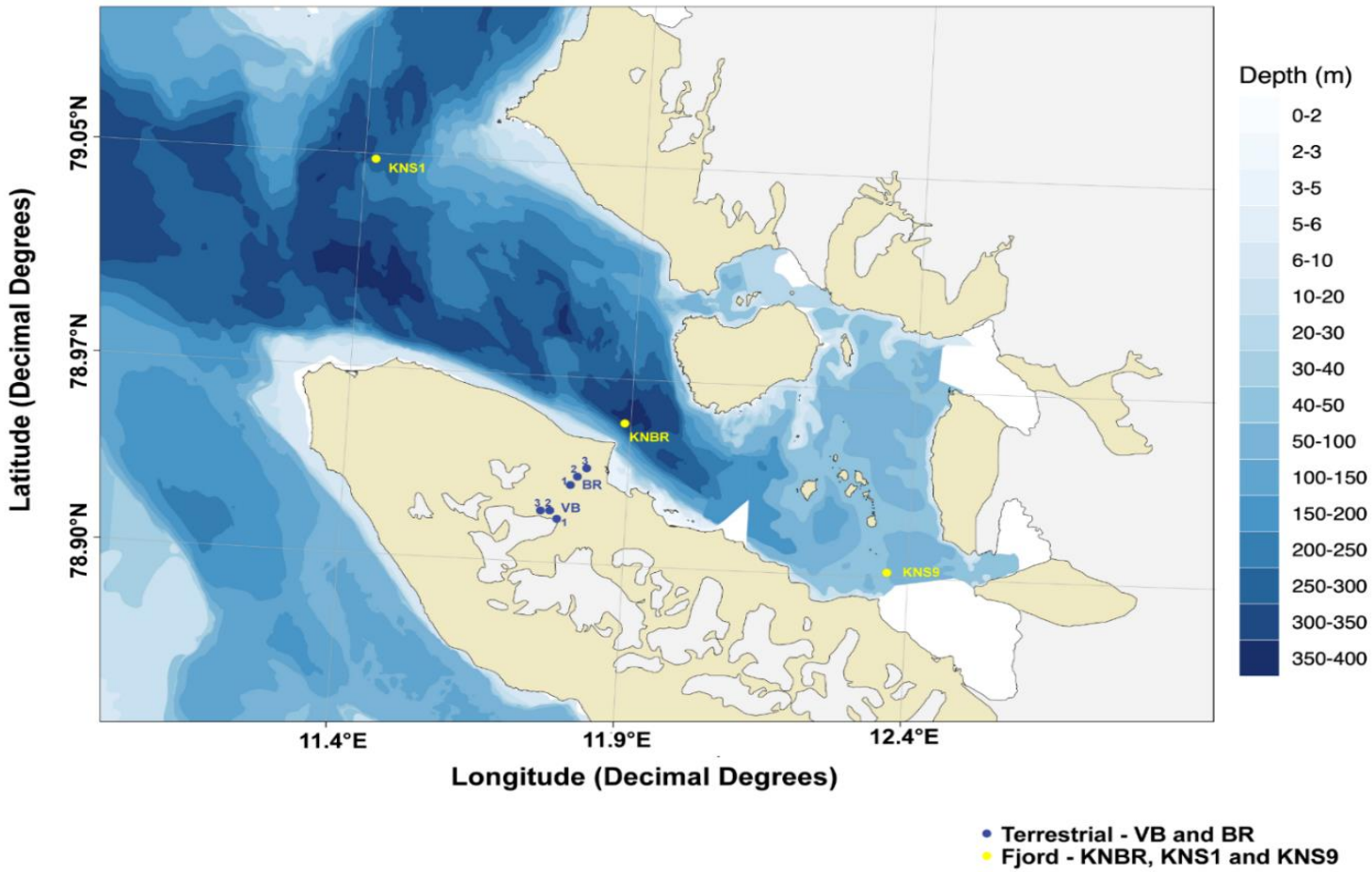

Fig. 3.10. Map of the glacierized terrestrial regions and Kongsfjorden with the sediment sampling points (Vestrebroggerbreen (VB) glacier snout, foreland (BR), and fjord (KNBR, KNS1, and KNS9) marked (Vihtakari, 2020). (*https://github.com/MikkoVihtakari/PlotSvalbard*

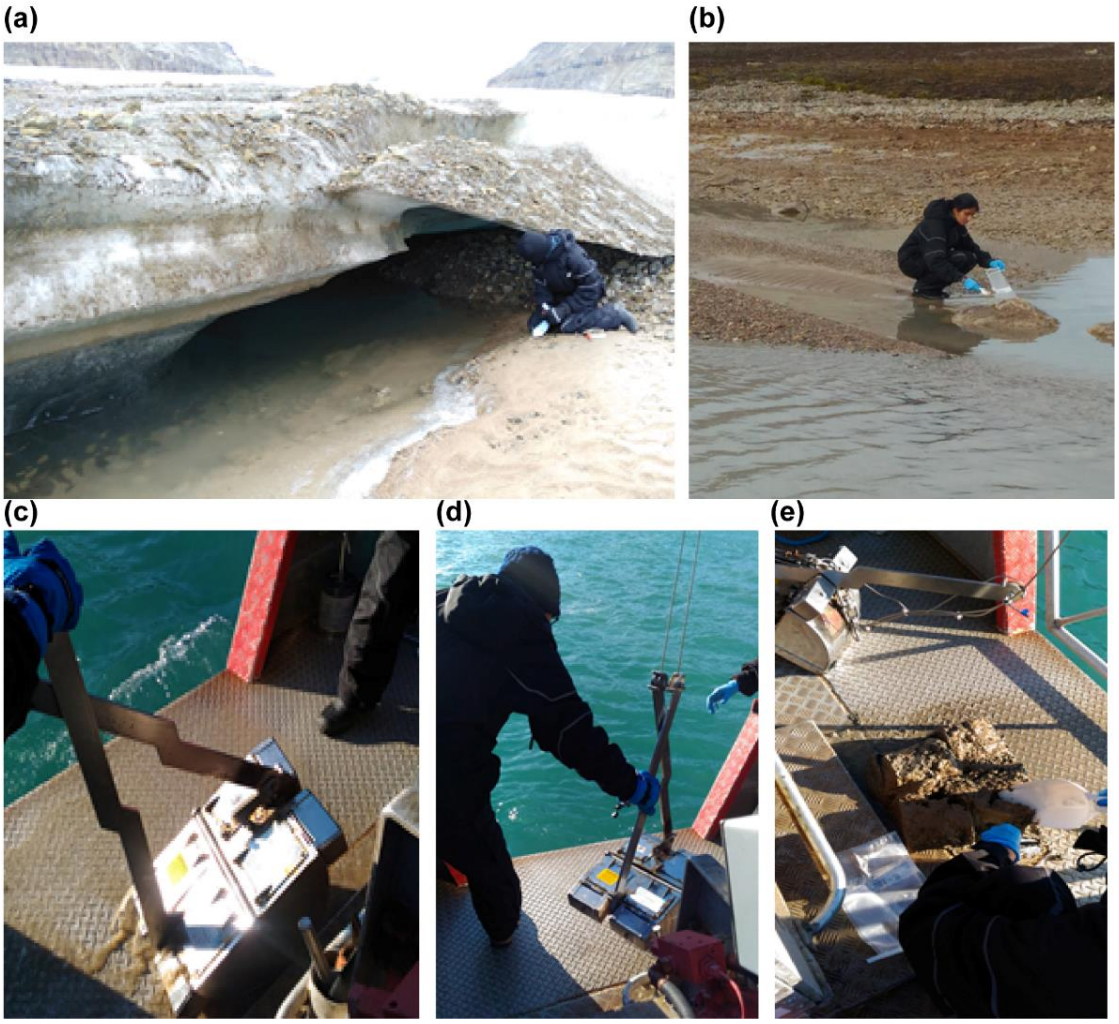

Fig. 3.11 (a) Vestrebroggerbreen glacier snout sediment sampling, **(b)** Bayelva sediment sampling, **(c)**, **(d),** and **(e)** fjord sediment sampling using a Van veen grab deployed from *MS Teisten*

3(b). 2.2. Chemical analysis of sediment samples

The pH of the sediment pore water was measured by centrifuging the sediment sample and measuring the pH of the supernatant water using Cyberscan pH meter 510 (Eutech Instruments, Thermo Fisher Scientific, USA). Water content (5g of each sample) was measured as gravimetric weight loss after drying the sediment at 105 °C until constant weight. Organic and inorganic carbon were analyzed as per Winkelmann and Knies (2005) using a PRIMACS^{MCS} TOC analyser (SKALAR, Netherlands). Sediment samples were analyzed for trace elements using an Inductively-coupled Plasma Mass Spectrometer (Thermo iCAP Q ICP-MS, Thermo Fisher Scientific, USA). Sample preparation involved microwave digestion as detailed by Lu et al. (2013) and the USEPA method 3051. In brief, 0.1g each of the oven-dried sediment samples were extracted in Conc. HNO_3 and Conc. HCl mix (7ml HNO_3 and 3mL HCl) and digested using microwave digester (Multiwave Go, Anton Paar, Austria). After cooling, the samples were filtered followed by a final dilution of 25 times with MilliQ. For the Total Mercury analysis, Direct Mercury Analyser (Duel cell-DMA 80, Milestone, Italy) was used following the method as per USEPA-7473. Briefly, 0.25 g each of the fully dried sediment samples were analyzed without any chemical pretreatment following the method based on thermal decomposition, amalgamation, and atomic absorption spectrometry (AAS). Absorbance (peak height or peak area) was measured at 253.7 nm as a function of mercury concentration.

3(b). 2.3. Total microbial abundance

Total sediment microbial count was carried out according to Epstein et al. (1997) using 4, 6-diamidino-2-phenylindole (DAPI) with minor modification. In brief, 0.5 g of sediment in 50 mL of filter-sterilized saline (0.9%) was vortexed for 10 min followed by ultra-probe sonication (at 40% speed over 80 sec with interruption of 2-4 times) using an Ultrasonic homogenizer (Model 3000, Biologics, Inc, USA) for dislodging cells from sediment particles. A 2mL aliquot of the sample was then stained with DAPI following the procedure described in **section 3(a). 2.4.**

3(b). 2.4. Isolation and identification of retrievable heterotrophic bacteria

For the bacterial retrieval, the sediment sample suspension was prepared by sonicating 5g of sediment in 45mL of filter-sterilized saline (0.9%). After serial dilutions (10^{-2} and 10^{-4} dilutions), the sediment suspension (100µL) was plated onto solid R2A media (1/2 strength), Antarctic Bacterial Medium (ABM), Tryptone Soy Agar (TSA) ($1/10^{th}$ strength), Actinomycete isolation agar (AIA) and Zobell Marine Agar (ZMA) medium ($1/4^{th}$ strength). The plates were then incubated at 4 ˚C and 20 ˚C for 2-3 weeks. The bacterial isolation, purification, genomic DNA extraction, Rep-PCR amplification for screening bacterial isolates, 16SrRNA gene amplification, sequencing, and 16S rRNA gene nucleotide sequence identification was carried out as discussed in the previous **section 3(a). 2.5.** The identified 16S rRNA gene sequences (104 sequences) were submitted in GenBank under the accession numbers **MN080149-MN080222**, **MT309496-MT309525**.

3(b). 2.5. Bacterial community analysis using amplicon sequencing and downstream bioinformatics processing

Environmental DNA from the sediment samples (0.5mg of sediment) was extracted using the DNeasy Power Soil kit (Qiagen, USA). The DNA concentration measurement, purity checking followed by sequencing on the HiSeq platform (Illumina, USA) using Pro341F/Pro805R primers were detailed in the **section 3(a). 2.6**. The raw nucleotide sequences were deposited in the NCBI Sequence Read Archive (SRA) database under the SRA accession number: **PRJNA475645**.

The downstream processing of raw DNA sequences was carried out according to the protocols detailed by Bolyen et al. (2019) using the QIIME2 software. The demultiplexed raw sequence datasets were trimmed for the removal of 16S rRNA gene primers and multiplexing adapters by using the QIIME2 Cutadapt plugin. Further, the sequences were subjected to denoising, dereplication, and chimera filtering using the DADA2 denoise-paired method of QIIME2. The clustering of reads into features was performed against the SILVA_132 marker gene reference database (Quast et al., 2012) by training the database using the q2-feature-classifier plugin. Unique representative features/OTUs from each sample were further subjected to taxonomic classification

using the classify-consensus-vsearch method in q2-feature-classifier plugin with a 0.99% identity threshold. This was followed by taxonomy-based filtering to remove the sequences representing chloroplasts and mitochondria using the q2-taxa plugin and rarefaction of the OTU counts (rarefaction depth-9458, 10 iterations) before the downstream diversity analysis. The alpha diversity estimation was carried out using the q2-diversity plugin.

3(b). 2.6. Community-level physiological profiling using EcoPlate

Biolog EcoPlate™ (Biolog Inc., USA) was used to evaluate community-level metabolic responses. The EcoPlate™ contains three replicate wells of 31 carbon substrates (Garland and Mills 1991) and a control well (A1) with no added carbon substrate. The 31 substrates used in the EcoPlate™ represented carbohydrates, polymers, carboxylic acids, amino acids, amines, and amides. Carbon substrate utilization by microbes is indicated by the respiration-dependent reduction of the tetrazolium dye leading to a purple coloration which was quantified spectrophotometrically using a microplate reader (Synergy 2-multi mode microplate reader, BioTek Instruments, USA). Preparation of sediment samples for EcoPlate™ analysis was performed as detailed by Sinha et al. (2019) In brief, the sediment samples (10 g) were homogenized in 20 ml of sterile 0.9% saline using a sonicator probe (Ultrasonic homogenizer 3000, Biologics Inc., VA, USA) with a 4.8 mm microtip vibrating at 190 μm amplitude at 50% for 2 min. Each turbid supernatant containing suspended microorganisms was adjusted to an optical density of 0.25 - 0.35 at 420 nm with saline. Aliquots of 150 μL were added per well and the EcoPlates were incubated at both 4 ˚C and 20 ˚C to understand the influence of temperature on the metabolic potential of the sediment community. The intensity of color development was measured at a wavelength of 595 nm for 240 hours (24 h time interval). The most intensive carbon substrate metabolism was observed at 192 h for 4 ˚C and 216 h for 20 ˚C incubation. The results were expressed as Average Well Color Development (AWCD) and the Shannon-Wiener (H) and Richness (Rs) indices were computed. The AWCD was evaluated according to Garland and Mills (1991) following formula:

$$\mathbf{AWCD=\Sigma(OD_i)/31};$$

where OD_i= C-R. C is the optical density measurement within each well and R is the absorbance value of the control well. The primary data were further normalized following the equation (C-R)/AWCD separately for each substrate using respective AWCD values. For the percentage utilization of each substrate category, the normalized values for each category were divided by the **Σ**(C-R)/AWCD values.

The Shannon-Wiener (H) index was calculated using the formula:

$$\mathbf{H= -\Sigma pi(lnpi)}$$

where pi is the ratio of the activity on each substrate (OD_i) to the sum of activities on all substrates **Σ**OD_i (Gomez et al., 2006). Richness index (R) is the number of substrates metabolized by the microbes in each sample.

3(b). 2.7. Understanding the growth yields of selected retrieved isolates of terrestrial and marine origin towards different carbon substrates

While the total sediment community metabolic profile was obtained from Ecoplate assay, it is imperative to understand how individual retrieved bacteria from terrestrial and marine samples could respond to different ecologically important substrates. For this purpose, from a total of 27 different species retrieved from the terrestrial sediments and 15 different species retrieved from the fjord sediments, we chose a total of 20 different species- 10 belonging to the terrestrial sediments and 10 from the fjord sediments. Isolates retrieved from both 4 °C and 20 °C incubation were chosen based on their relative abundance. The fully grown bacterial cultures in the broth media were centrifuged (5000 rpm, 15 min) and the resulting pellet was washed multiple times with 0.9% saline. The bacterial inoculum thus obtained was fixed to an optical density (OD) of 0.2 at 600nm and the corresponding number of bacterial cells was measured using DAPI staining (protocol same as detailed in **section 3(a). 2.4**). The substrate utilization experiment was carried out in microplates wherein a 10µL culture aliquot (with fixed

OD of 0.2) was added to each well which was preloaded with 140 µL of a substrate minimum medium mix. Substrates tested included carbohydrates (D-cellobiose, sucrose, D-mannitol, erythritol, D-lactose, D-glucose, N-acetyl-D-glucosamine, and D-xylose), polymers (Tween 80, cellulose), amino-acids (L-phenyl alanine, L-serine, DL-Asparagine, L-glutamic acid), and carboxylic acids (D-malonic acid, D-galacturonic acid, p-aminobenzoic acid, DL-malic acid, and sodium pyruvate). Substrates were chosen based on the Biolog EcoPlate™ substrate list with a few additional substrates included and were filter-sterilized before use. Each substrate was added to a Minimal media (MM composition: KH_2PO_4 - 0.5 g; NH_4C1 - 0.4 g; $MgSO_4.7H_2O$ - 0.4 g; NaC1 - 0.4g; $CaC1.2H_2O$ - 0.05 g; $Na_2S_20_3.5H_20$ - 0.2 g; Fe-citrate - 0.005 g; trace element solution (10 ml); MilliQ to 1000 ml. The pH was adjusted to 5.6. The trace element solution has the following composition: $ZnSO_4.7H_20$ - 10 mg; $MnC1_2.4H_2O$ - 3 mg; H_3BO_3 - 30 mg; $CoC1_2.6H_20$ - 20 mg; $CuC1_2.2H_2O$ - 1 mg; $NiC1_2.6H_20$ - 2 mg; $Na_2Mo0_4.2H_2O$ - 3 mg; MilliQ water 1000 ml; as per Freese et al., 2010 and Pfennig, 1974) to produce a final concentration of 50 mg/L (Freese et al., 2010). The experiment was carried out in triplicates with controls comprising of MM with bacteria and without substrates, MM with substrates and without bacteria, and MM without bacteria and substrates. The experiment was tested at both 4 °C and 20 °C temperature incubations to check the effect of temperature on bacterial growth by the utilization of different carbon substrates. Absorbance (OD 600 nm) was measured for 15 days at regular intervals of 0, 3, 6, 9, 12, and 15 days.

For each of the selected bacterial isolates, the optimum temperature and temperature range for growth was determined on diluted R2A broth media (50% w/v of the original strength of 3.12 gm/L) for terrestrial isolates and diluted ZMB (25% w/v of the original strength of 40.25 gm/L) for fjord isolates at different temperature incubations (4, 10, 15, 20, 25, 30, and 35 °C) by measuring optical density at 600 nm.

3(b). 2.8. Statistical analysis

The influence of geochemical properties in explaining the bacterial community structure associated with the terrestrial and fjord sites was done by Canonical Correspondence Analysis (CCA) (using Bray Curtis dissimilarity) using CANOCO 4.56 statistical software. Pearson linear correlation was calculated using OriginPro 9.0 software to measure the correlation between geochemical properties and total bacterial community structure, bacterial community and Ecoplate substrate utilization as well as between geochemical properties and Ecoplate substrate utilization. Principal component analysis (PCA) was performed using the Canoco 4.56 package to ordinate the strains depending on the carbon sources they were able to metabolize. One-way Analysis of Variance (ANOVA) was performed for examining significant differences between the metabolic profiles of terrestrial and fjord sediment communities as well as between the retrievable bacterial isolates of the two systems, at 4 °C and 20 °C incubations.

3(b). 3. Results

3(b). 3.1. Geochemical properties of sediment samples

The overlying water temperature for the terrestrial sediments varied between 0.9 °C-1.3 °C and salinity was 0.08 PSU, while that of the fjord sediments varied between 3.8 °C-5.9 °C and 34.3 - 34.9 PSU (**Table 3.9**). The highest value of sediment pore water pH was observed in VB and BR sediments (~8.4) while the pH of the fjord sediment pore water ranged from 7.9 - 8.1 (**Table 3.9**). The total organic carbon (TOC) content among the terrestrial and fjord sediments varied between 1.2 - 2.9% with the highest value recorded in the outer fjord sediment (KNS1) and the lowest value noted in the foreland sediment (BR).

A reduction in the concentration of TOC was observed from the outer to the inner fjord sediments while an increase in Inorganic carbon (IC) concentration was noted towards the inner fjord sediments (**Table 3.9**). The highest values of Mn (362.0 mg/Kg), Co (13.2 mg/Kg), Cu (33.6 mg/Kg), and Zn (223.9 mg/Kg) was observed in BR while the highest concentration of Ni (40.8 mg/Kg) and Cd (0.3 mg/Kg) was found in VB. Similarly, the outer fjord sediment (KNS1) exhibited the highest concentrations of Pb

(7.9 mg/Kg) and Hg (90.9 µg/Kg) (**Table 3.9**). We could also observe significant correlations between the geochemical factors such as Ni and IC ($p<0.005$), TOC and Hg ($p<0.05$), Mn and Co ($p<0.005$) as well as Hg and Pb ($p<0.01$) (**Table 3.10**).

3(b). 3.2. Total microbial counts, retrievable heterotrophic bacterial counts, and phylogeny of cultivated bacterial isolates

Total microbial counts (TMC) in the sediment samples ranged between 10^{6-8} cells/g. The lowest count ($4.3x10^6$ cells/g) was observed in the terrestrial sediment BR while the highest count ($4.9x10^8$ cells/g) was recorded in the outer fjord sediment KNS1 (**Table 3.9**).

The total isolated heterotrophic bacterial count was calculated for both 4 °C and 20 °C incubations. Half-strength R2A media yielded the highest retrievable count for the terrestrial sediments at both the incubation temperature (4 °C: VB - $9.1x10^3$ CFU/g and BR - $3.2\ x10^3$ CFU/g and 20 °C: VB - $2.8x10^3$ CFU/g and BR - $1.1x10^3$ CFU/g) while quarter-strength ZMA yielded the highest number of isolates for the fjord sediments at both temperatures (4 °C: $1.6x10^5$ - $2.5x10^5$ CFU/g and 20 °C: $7.7x10^4$ - $1.1x10^5$ CFU/g) (**Table 3.9**).

106 and 64 bacterial isolates were retrieved (based on colony characteristics) from terrestrial and fjord sediments respectively from 4 °C incubation. Similarly, a total of 25 and 60 bacterial isolates were retrieved from 20 °C incubation. Pigmentation was observed for more than half of the total terrestrial and fjord sediment retrievable bacterial fraction. Based on the banding pattern obtained by rep-PCR, 109 bacterial isolates with unique banding patterns were selected for further identification. The results from 16S rRNA gene sequencing based on a similarity cut-off > 98.7% revealed the presence of 27 different bacterial species in terrestrial sediments (VB and BR) and 15 in the fjord sediments (KNBR, KNS1, and KNS9).

Table 3.9.

Geochemical properties, total microbial counts (TMC), and retrievable heterotrophic bacterial counts (RHBC) in the sediments.

Parameters	**Glacier sediments**		**Fjord sediments**		
	VB	**BR**	**KNBR (Depth-126m)**	**KNS1 (Depth-240m)**	**KNS9 (Depth-46.5m)**
Sediment porewater pH	8.4	8.4	7.9	8.0	8.1
Water content (%)	20.4	15.4	18.9	20.5	20.9
TOC (%)	1.9	1.2	2.3	2.9	1.3
IC (%)	6.3	1.3	2.8	2.2	3.1
Mn (mg/Kg)	205.3	362.0	250.5	314.1	332.6
Ni (mg/Kg)	40.8	16.1	20.8	21.3	26.6
Co (mg/Kg)	8.3	13.2	9.4	11.7	11.9
Cu (mg/Kg)	12.6	33.6	19.1	21.2	19.1
Zn (mg/Kg)	166.7	223.9	152.9	136.1	117.3
Cd (mg/Kg)	0.3	0.1	0.2	0.2	0.1
Pb (mg/Kg)	3.9	2.9	3.0	7.9	2.6
Hg (µg/Kg)	44.8	18.2	38.6	90.9	22.1
TMC (cells/g)	6.2×10^{6}	4.3×10^{6}	2.2×10^{8}	4.9×10^{8}	4.7×10^{8}
RHBC at 4°C (CFU/g)	$9.1\times10^{3*}$	$3.2\times10^{3*}$	$1.6\times10^{5**}$	$2.5\times10^{5**}$	$2.5\times10^{5**}$
RHBC at 20°C (CFU/g)	$2.8\times10^{3*}$	$1.1\times10^{3*}$	$7.7\times10^{4**}$	$1.1\times10^{5**}$	$8.5\times10^{4**}$

** On half-strength R2A medium; ** On quarter-strength ZMA*

Eight bacterial sequences retrieved (7 from the terrestrial sediments and 1 from the fjord sediments) exhibited < 98.7% sequence similarity with the closest type strain in the EzBioCloud database indicating their novelty (Table 3.11).

The dominant bacterial isolates from the terrestrial and fjord sediments were also tested to estimate their optimal temperature as well as temperature range for growth. The majority of the bacterial isolates (about 85%) were psychrotolerant (capable of growth close to 0°C but with an optimum growth temperature of > 20 °C) while 3 isolates fit into the criteria for true psychrophiles (optimal temperature for growth at about 15 °C or lower and a maximal temperature for growth at about 20 °C). The psychrophilic isolates were closely related to the species *Polaromonas glacialis, Cryobacterium psychrotolerans,* and *Polymorphobacter fuscus,* all of which were isolated from the terrestrial sediments in our study.

The bacterial isolates from the terrestrial and fjord sediments belonged to 4 phyla - Proteobacteria (constituted by class γ-proteobacteria and α-proteobacteria), Bacteroidetes, Actinobacteria, and Firmicutes (**Fig. 3.12**). Phylum Actinobacteria dominated the terrestrial sediments at both 4 °C (58% of the total isolates) and 20 °C (38.5% of the total) incubation. The other dominant phyla isolated from the terrestrial sediments included Proteobacteria and Bacteroidetes. Bacteroidetes accounted for 13.4% of the total isolates from 4 °C and 33.3% of the total from 20 °C incubation. Class γ-proteobacteria was dominant at 4 °C (27.5% of the total) while class α-proteobacteria was dominant at 20 °C (25.6% of the total) (**Fig. 3.12**). The phylum Actinobacteria was represented by 5 different genera, of which the genus *Cryobacterium* was dominant with 7 different species (**Table 3.12a and 12b)**. The class γ-proteobacteria was represented by the genera *Janthinobacterium, Herminiimonas, Polaromonas, Hydrogenophaga* (order Betaproteobacteriales), and *Pseudomonas* (order Pseudomonadales). The genus *Flavobacterium* solely represented the phylum Bacteroidetes while the genus *Polymorphobacte*r represented the class α-proteobacteria. The dominant species isolated from the terrestrial sediments from 4°C incubation were *Cryobacterium psychrotolerans* (27.9% of the total species), *Janthinobacterium lividum* (19.5%), *Cryobacterium roopkundense* (19.1%), *Flavobacterium degerlachei* (7.6%), and *Polaromonas glacialis* (4.6%). Similarly, the

dominant species isolated from 20 °C incubation were *Polymorphobacter fuscus* (25.6% of the total species), *Flavobacterium degerlachei* (23%), *Cryobacterium luteum* (17.9%), *Flavobacterium omnivorum* (10.3%), and *Cellulomonas cellasea* (7.7%).

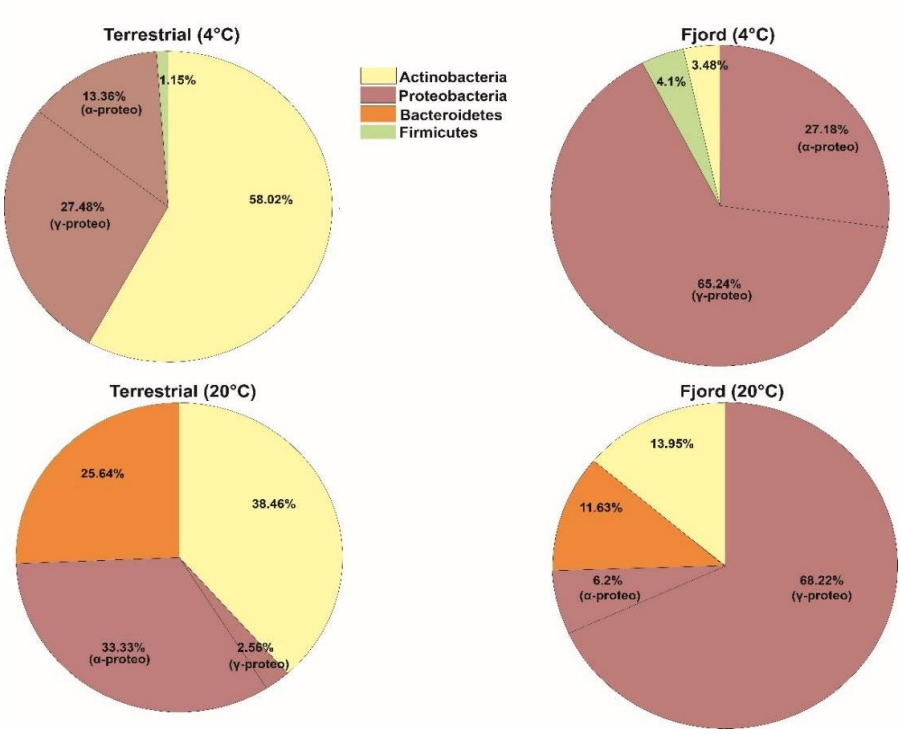

Fig. 3.12. Percentage composition of retrievable bacteria belonging to the phylum Proteobacteria, Actinobacteria, Bacteroidetes and Firmicutes present in the terrestrial and fjord sediments from 4 °C and 20 °C incubation. Further class level abundance of the dominant phylum Proteobacteria is also shown in each pie diagram since the abundance of α- and γ-proteobacteria varied between the terrestrial and fjord sediments.

γ-proteobacteria dominated the retrievable bacterial fraction in the marine fjord sediment samples at both 4 °C (65.2% of the total) and 20 °C (68.2% of the total), with the presence of 4 genera (*Psychrobacter, Oceanisphaera, Shewanella, and Photobacterium*) and 6 different species **(Fig. 3.12)**. Bacterial species belonging to the phylum Actinobacteria were retrieved only from the inner fjord sediment KNS9. The dominant species retrieved from the fjord sediments at 4 °C incubation were *Psychrobacter fozii* (26.3% of the total species), *Psychrobacter cryohalolentis* (17.8%), *Psychrobacter glaciei* (16.6%), and *Flavobacterium degerlachei* (16.5%). Similarly, *Psychrobacter glaciei* (41.9%) and *Psychrobacter fozii* (13.95%) dominated the retrievable fraction at 20 °C incubation. The genus *Flavobacterium* solely represented the phylum Bacteroidetes while the genus *Planococcus* and *Planomicrobium* represented the phylum Firmicutes.

The presence of the genus *Flavobacterium* (phylum Bacteroidetes) with *Flavobacterium degerlachei* being the most common representative was retrieved from both terrestrial and fjord sediments from both temperature incubations

Table 3.10.
Significant correlations between geochemical parameters obtained by Pearson linear correlation.

Factors	**r value obtained**	**Level of significance**
Between geochemical parameters		
IC%-Ni	0.985993	**
TOC%-Hg	0.931025	*
Mn-Co	0.995782	***
Pb-Hg	0.973018	**
Zn-water content%	-0.91471	*

*p value of 0.05 represented as *, p value of 0.01 (**) and p value of 0.001 (***)*

Table 3.11.
List of retrievable heterotrophic bacterial isolates from the terrestrial and Kongsfjorden sediments with their taxonomic affiliation and closest type strain description.

Strain ID	Closest relative type strain	% similarity	Isolation source of type strain/description
	Terrestrial sediments		
4VBSed**A**2	*Arthrobacter ginsengisoli* (KF212463)	99.25	From ginseng field soil, Republic of Korea
20BRSed**AA**1	*Arthrobacter pascens* (X80740)	98.54	-
20VBSed**T**4	*Cellulomonas bogoriensis(AXCZ01000188)*	96.59	From lake sediment, Kenya. An alkaliphilic, slightly halotolerant, chemo-organotrophic bacteria
4BR1Sed**T**3	*Cellulomonas cellasea (X83804)*	96.49	From soil in Japan
4VBSed**R**8	*Cryobacterium arcticum (GQ406814)*	99.4	Psychrotolerant bacteria, from soil sample from north-east Greenland.
4BR1Sed**Z**5	*Cryobacterium aureum (JF267311)*	99.7	From Xinjiang No. 1 Glacier in China
4VBSed**R**7	*Cryobacterium flavum(jgi.1076268)*	99.3	From Xinjiang No. 1 Glacier in China
4BR1Sed**Z**10	*Cryobacterium levicorallinum(JF267312)*	99.85	From Xinjiang No. 1 Glacier in China
20VBSed**R**2	*Cryobacterium luteum (jgi.1076264)*	99.54	From Xinjiang No. 1 Glacier in China
4BR2Sed**Z**2	*Cryobacterium psychrotolerans(jgi.1076200)*	98.76	From Xinjiang No. 1 Glacier in China
4VBSed**R**1	*Cryobacterium roopkundense(EF467640)*	99.41	Psychrophilic bacteria from soil from glacial Lake Roopkund, Himalayan mountain range
4VBSed**T**3	*Flavobacterium degerlachei (jgi.1107974)*	99.13	From microbial mats in Antarctic lakes
20VBSed**R**5	*Flavobacterium omnivorum (AF433174)*	99.77	From Xinjiang No. 1 Glacier in China
4VBSed**AA**3	*Flavobacterium piscis (LVEN01000016)*	97.76	From diseased rainbow trout

4BR2Sed**A**3	*Flavobacterium sinopsychrotolerans (FJ654474)*	99.7	From frozen soil, China No. 1 glacier
4BR1Sed**T**4	*Herminiimonas arsenitoxidans (LT671418)*	96.97	From heavy metal -contaminated soil, oxidize arsenite
20BRSed**R**2	*Hydrogenophaga palleroni (BCTJ01000079)*	98.29	Yellow-pigmented hydrogen-oxidizing species
4BR1Sed**T**2	*Janthinobacterium lividum (Y08846)*	99.53	Common in soil and water in cold-temperate regions, produce violacein pigment, biofilm formation
4BR2Sed**Z**1	*Marisediminicola antarctica (GQ496083)*	99.78	From intertidal sediment sample from east Antarctica
4BR2Sed**T**5	*Polaromonas cryoconiti (HM583567)*	99.09	From alpine glacier cryoconite
4BR2Sed**T**2	*Polaromonas eurypsychrophila (KP013181)*	99.41	From ice core from Muztagh Glacier, Tibetan Plateau
4VBSed**T**8	*Polaromonas glacialis (HM583568)*	99.48	Psychrophilic bacteria, isolated from alpine glacier cryoconite
20BRSed**Z**4	*Polymorphobacter fuscus (KF737330)*	99.48	From permafrost soil of Kunlun mountains, Qinghai-Tibet plateau.
4BR3Sed**R**8	*Pseudomonas mandelii (BDAF01000092)*	99.77	From natural mineral waters
4BR1Sed**AA**5	*Pseudomonas silesiensis (KX276592)*	97.75	From a biological pesticide sewage treatment plant
4VBSed**T**1	*Salinibacterium xinjiangense (DQ515964)*	99.7	Psychrophilic bacteria, isolated from the China No. 1 glacier.
4BR1Sed**A**3	*Trichococcus collinsii (AJ306612)*	99.8	From a gas-condensate-contaminated soil in Colorado
	Fjord Sediments		
4KNS9Sed**R**4	*Arthrobacter humicola (AB279890)*	99.71	From paddy soil, Japan
4KNS1Sed**Z**1	*Flavobacterium degerlachei (jgi.1107974)*	99.71	From microbial mats, Antarctic lakes
4KNS1Sed**A**3	*Flavobacterium frigoris (jgi.1107976)*	99.39	From microbial mats, Antarctic lakes

20KNS1Sed**R**1	*Oceanisphaera ostreae (HQ340607)*	99.5	From seawater of an oyster farm, S.Korea
4KNS9Sed**A**1	*Paeniglutamicibacter antarcticus (AM931709)*	98.92	From Antarctic marine sediment
4KNS9Sed**R**2	*Photobacterium frigidiphilum (AY538749)*	99.71	From deep-sea sediments in the western Pacific Ocean
4KNS9Sed**Z**1	*Planococcus halocryophilus (ANBV01000012)*	99.79	From permafrost active-layer soil from the Canadian high Arctic, growth at -15°C and high salinity
4KNS9Sed**Z**4	*Planomicrobium flavidum (FJ265708)*	99.28	From a marine solar saltern
4KNS9Sed**A**2	*Psychrobacter cryohalolentis (CP000323)*	99.79	From Siberian permafrost, growth at -10 to 30
4KNS1Sed**AA**3	*Psychrobacter fozii (AJ430827)*	99.64	From Antarctic coastal marine environments
4KNS1Sed**A**4	*Psychrobacter glaciei (FJ748508)*	99.86	From ice core from Austre Lovénbreen, Ny-Ålesund, Svalbard
20KNBRSed**R**2	*Psychrobacter nivimaris (AJ313425)*	100	From South Atlantic (Antarctica), particle attached bacteria
20KNBRSed**Z**3	*Paracoccus sediminilitoris (MH491014)*	98.94	From a tidal flat sediment, East China Sea
20KNS9Sed**AA**1	*Nesterenkonia aurantiaca (HG795012)*	99.41	From a soil sample from Antarctica, haloalkaliphilic
4KNS1Sed**R**1	*Shewanella marinintestina (AB081757)*	98.01	From sea-animal intestines, ability to produce eicosapentaenoic acid

*One representative isolate (having the highest base-pair length) for each species was described in the table. Sequences with similarity level < 98.7% mostly likely represent novel species that are underlined. Strain IDs begin with numeric 4 or 20 representing the temperature of incubation, the alphabets written in bold indicates the media used for isolation (**A** - Antarctic Bacterial Medium, **R** - R2A Agar (1/2 strength), **Z** - Zobell Marine Agar (1/4 strength), **T** - Tryptic Soya Agar (1/10 strength), **AA** - Actinomycete Isolation Agar). For eg. **4**VBSed**A**2 is isolated from Antarctic Bacterial Medium at 4 °C incubation.*

Table 3.12a.

The relative abundance of heterotrophic bacterial genera retrieved from different growth media from terrestrial and fjord sediments at 4 °C incubation.

4 Degree Isolates	**Terrestrial**					**Kongsfjorden**				
	R2A	TSA	ABM	AIA	ZMA	R2A	TSA	ABM	AIA	ZMA
Cryobacterium	52.8	27.47	29.69	40	90	-	-	-	-	-
Flavobacterium	17.97	4.39	20.31	40	-	-	-	25.47	-	16.13
Janthinobacterium	22.47	-	-	-	-	-	-	-	-	-
Trichococcus	-	-	46.87	-	-	-	-	-	-	-
Cellulomonas	5.62	-	-	-	-	-	-	-	-	-
Salinibacterium	-	1.09	-	-	-	-	-	-	-	-
Marisediminicola	-	1.09	-	-	10	-	-	-	-	-
Polaromonas	-	18.68	-	-	-	-	-	-	-	-
Janthinobacterium	-	34.07	-	-	-	-	-	-	-	-
Cellulomonas	-	12.09	-	-	-	-	-	-	-	-
Herminiimonas	-	1.09	-	-	-	-	-	-	-	-
Arthrobacter	-	-	3.13	-	-	26	-	-	-	-
Pseudomonas	1.12	-	-	20	-	-	-	-	-	-
Psychrobacter	-	-	-	-	-	30	-	62.26	87.60	41.94
Paeniglutamicibacter	-	-	-	-	-	-	-	12.26	-	13.98
Shewanella	-	-	-	-	-	2	-	-	-	-
Oceanisphaera	-	-	-	-	-	12	-	-	-	-
Photobacterium	-	-	-	-	-	30	-	-	-	
Planococcus	-	-	-	-	-	-	-	-	12.40	16.13
Planomicrobium	-	-	-	-	-	-	-	-	-	11.83

Table 3.12b.

The relative abundance of bacterial genera retrieved from different growth media from terrestrial and fjord sediments at 20 °C incubation.

20 Degree Isolates	**Terrestrial**				**Kongsfjorden**				
	R2A	TSA	AIA	ZMA	R2A	TSA	ABM	AIA	ZMA
Cryobacterium	43.75	-	-	-	-	-	-	-	-
Flavobacterium	31.25	88.89	-	-	27.27	100.0	-	-	-
Cellulomonas	18.75	11.11	-	-	-	-	-	-	-
Arthrobacter	-	-	100.0	16.67	-	-	-	-	-
Polymorphobacter	-	-	-	83.33	-	-	-	-	-
Hydrogenophaga	6.25	-	-	-	-	-	-	-	-
Psychrobacter	-	-	-	-	68.18	-	-	88.89	73.68
Paeniglutamicibacter	-	-	-	-	-	-	100.0	-	-
Oceanisphaera	-	-	-	-	4.55	-	-	-	-
Nesterenkonia	-	-	-	-	-	-	-	11.11	-
Paracoccus	-	-	-	-	-	-	-	-	26.32

R2A - R2A medium (1/2 strength), TSA - Tryptone Soy Agar (1/10th strength), AIA - Actinomycete isolation agar, ZMA - Zobell Marine Agar medium (1/4th strength), and ABM - Antarctic Bacterial Medium

3(b). 3.3. Bacterial diversity as revealed by 16S rRNA gene amplicon sequencing

A total of 223613 sequences and 2376 OTUs were obtained in the present study using SILVA _132 database at 99% sequence similarity. The Good's coverage estimator of the OTUs ranged between 99.7 - 99.9% (**Table 3.13**), indicating sufficient sequence coverage in all the samples. It was found that 30914 sequences representing 1263 OTUs were not identified based on the user database and hence have been characterized as unassigned OTUs. The unassigned OTUs accounted for about 19.98% of the total sequences identified from sediments of VB, 25% of BR, 26.8% of KNBR, 2.3% of KNS1, and 8.3% of KNS9. 1103 identified bacterial OTUs were classified into 22 phyla, 42 classes, 74 orders, 119 families, and 142 genera. In total, Proteobacteria (avg 31.2%), Epsilonbacteraeota (avg 18.9%), and Bacteroidetes (avg 16.3%) dominated the overall bacterial community. A total of 10 archaeal OTUs (1611 sequences) were identified representing the classes Bathyarchaeia, Thermoplasmata, and Nitrososphaeria. However, the number of archaeal OTUs detected in the amplicon analysis was only 0.42% of the total OTUs recovered.

OTUs from Proteobacteria (18.9 - 45.75%) and Bacteroidetes (7.5 - 24.2%) dominated both the terrestrial and fjord sediments. Verrucomicrobia was most abundant in the terrestrial sediment BR (19.4%) while Cyanobacterial OTUs were only found in the VB sediment (6.6%) Similarly, Patescibacteria and Planctomycetes were detected only in terrestrial sediments (**Fig. 3.13**). Epsilonbacteraeota mainly resided in the outer fjord sediment KNS1 (50%) (**Fig. 3.13**) while Fusobacterial OTUs were observed only at station KNS9 (10.3%).

The relative abundance of the major (> 1% abundance), minor (0.1 - 1% abundance), and rare bacterial genera (< 0.1% abundance) are summarized in **Fig. 3.14**. The presence of the bacterial genus *Flavobacterium* was noted in all 5 sediment samples (**Fig. 3.14**), results being similar to the culture-based study. The relative abundance of Flavobacterial OTUs was found to be > 4% in all the samples except for KNBR sediment where the abundance was 0.69%. The dominant genera identified in the VB sediment were *Flavobacterium* (12%), *Rhodoferax* (9%), *WCHB1-12* (3.9%), *Pseudomonas* (3.9%), *Gemmatimonas* (3%), and *Phormidesmis* (2.9%). Similarly, in BR sediment, the dominant genera noted were *Luteolibacter* (19%), *Clostridium*

(7.7%), *Polaromonas* (5%), *Flavobacterium* (4%), *BSV13* (4.7%), and *Rhodoferax* (3%).

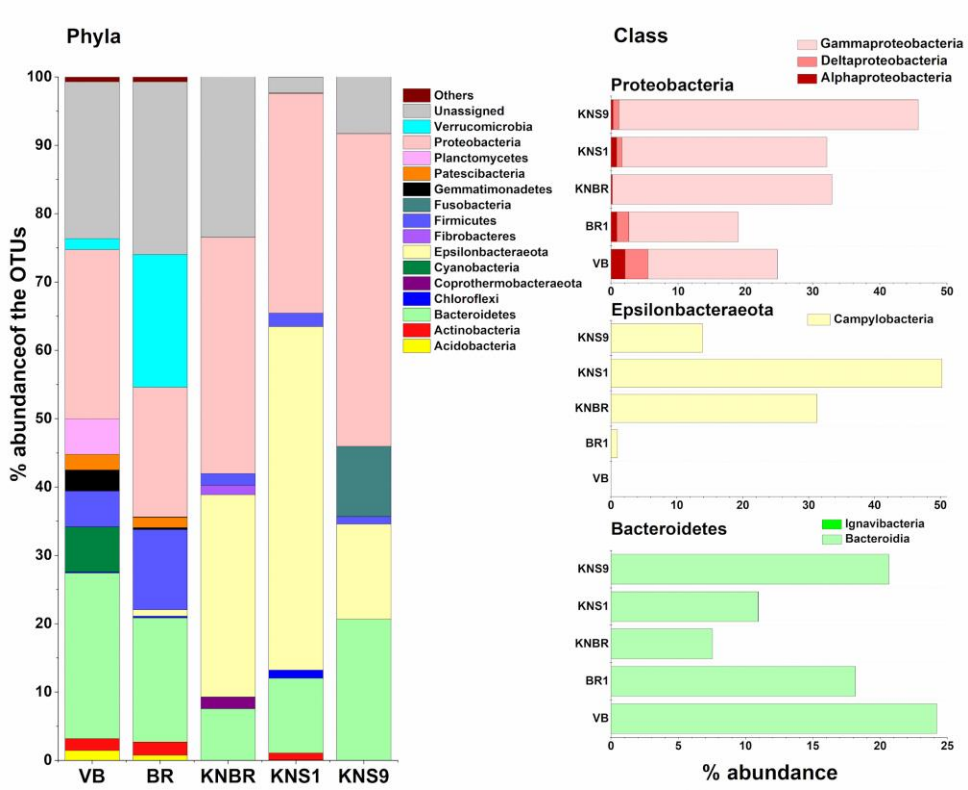

Fig. 3.13. The relative abundance of bacterial OTUs at the phylum level and the abundance of major bacterial classes in the amplicon - based culture-independent study.

The major bacterial genera in the fjord sediments were *Photobacterium* (23.6% - 41%), *Sulfurovum* (12.5% - 24%), *Sulfurimonas* (0.8% - 37.8%), *Flavobacterium* (0.7% - 13%), *Lutimonas* (1% - 3.5%), and *Psychromonas* (0.3% - 2.8%) (**Fig. 3.14**). The bacterial genera which were present exclusively in the terrestrial sediments VB and BR included *Polaromonas*, *Rhodoferax*, *Geobacter*, *Gemmatimonas*, *Sphingomonas*, *Massilia*, *BSV13*, *WCHB1-32*, and *Williamwhitmania*. Similarly, the bacterial genera present exclusively in the fjord sediments included *Marinifilum*, *Psychroserpens*, *Paraglaciecola*, and *Psychromonas* (**Fig. 3.14**).

There were a total of 56 minor bacterial genera (relative abundance between 0.1 - 1%) and 54 rare genera (relative abundance < 0.1%) present in the samples. VB sediment

was highly diverse in terms of minor and rare taxa (60.7% of total minor genera and 64.8% of the rare genera noted for VB sediment) while KNBR sediment represented the least number of minor (14.28%) and rare genera (1.85%) (**Fig. 3.14**).

Alpha diversity of bacterial communities from VB, BR, and fjord sediments was quantified based on species richness (total number of OTUs), richness estimators Chao1, diversity indices (Shannon index and Simpson's index), and phylogenetic diversity (PD). The Shannon diversity index was highest for VB sediment (8.1) while the lowest value was noted for KNS9 sediment (4.5). We could observe a reduction in the bacterial diversity and richness from the glacier to the fjord sediments (**Table 3.13**).

Table 3.13.
List of total sequences, OTUs, goods coverage, and various diversity indices for each sediment sample.

Sample	Total sequences	Total OTUs	Goods coverage	Shannon	Simpson	Chao1
VB	92598	1150	0.999	8.135	0.988	1244.721
BR	18233	612	0.997	7.935	0.990	638.775
KNBR	9458	150	0.999	5.376	0.941	150
KNS1	50046	302	0.999	5.296	0.944	322.539
KNS9	50056	283	0.999	4.511	0.866	298.545

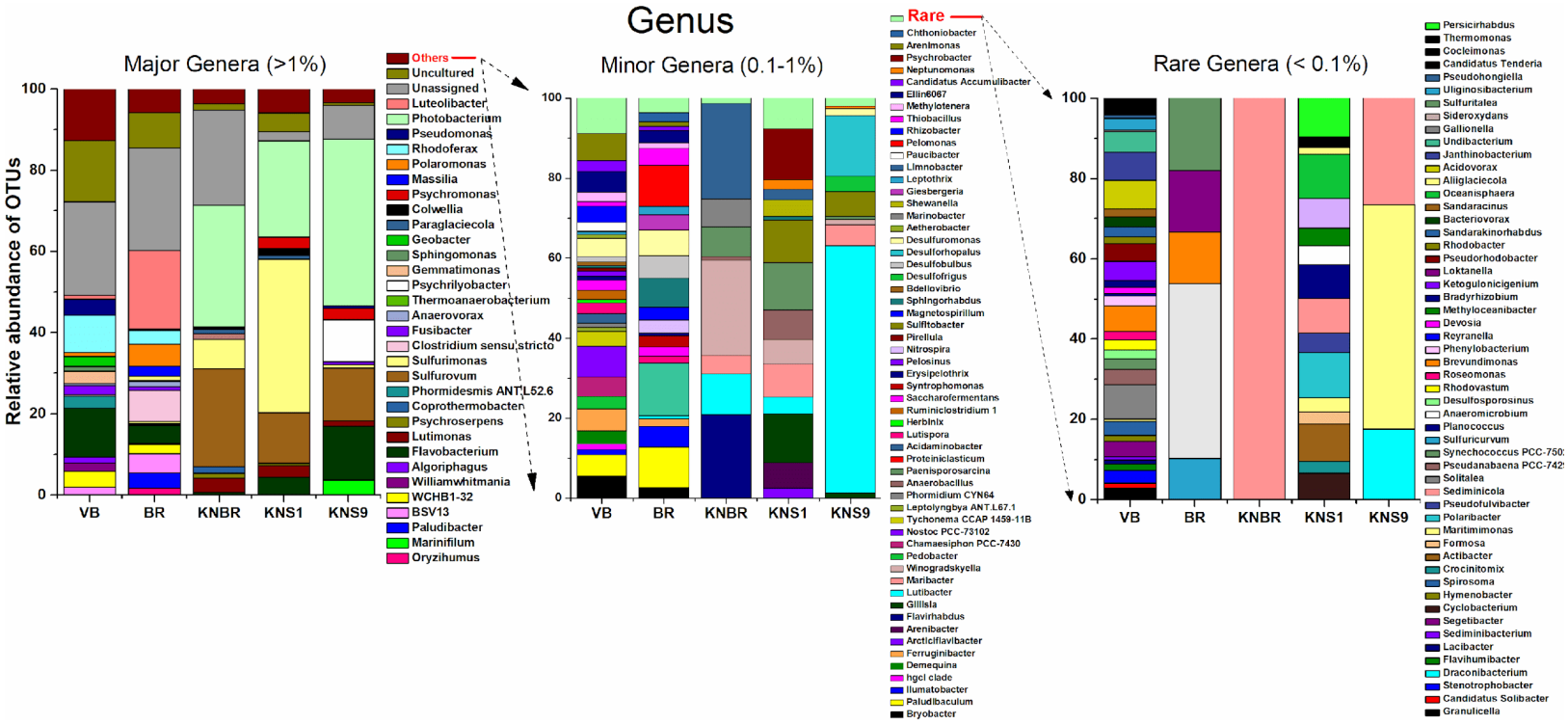

Fig. 3.14. The relative abundance of the major bacterial genera (> 1% of the total abundance), minor genera (0.1 - 1% of the total abundance), and rare genera (< 0.1% abundance) observed in the study.

3(b). 3.4. Bacterial community structure between the sampling sites and their correlation with the sediment geochemical properties

Canonical Correspondence Analysis showed that the total eigenvalue obtained as the summation of Axis 1 to 4 was 0.79. Axis 1 and Axis 2 accounted for 71.9% of the cumulative percentage variance observed in the relationship between species and the various environmental factors tested (**Fig. 3.15**). Among the various environmental factors tested in CCA, pore water pH showed significant influence on the species distribution (p-value <0.005) explaining about 39% of the total variance by all variables. We could also observe a close association of IC (Inorganic Carbon) and trace metals such as Ni and Cd with the terrestrial VB bacterial community while Zn and sediment pore water pH exhibited a positive correlation with the BR community. Similarly, the close association of Pb, Hg, TOC, and water content with the fjord sediment communities was noted (**Fig. 3.15**). Furthermore, significant correlations were observed between different bacterial taxa and also between bacterial taxa and the geochemical properties of sediments as per Pearson linear correlation (**Table 3.14**).

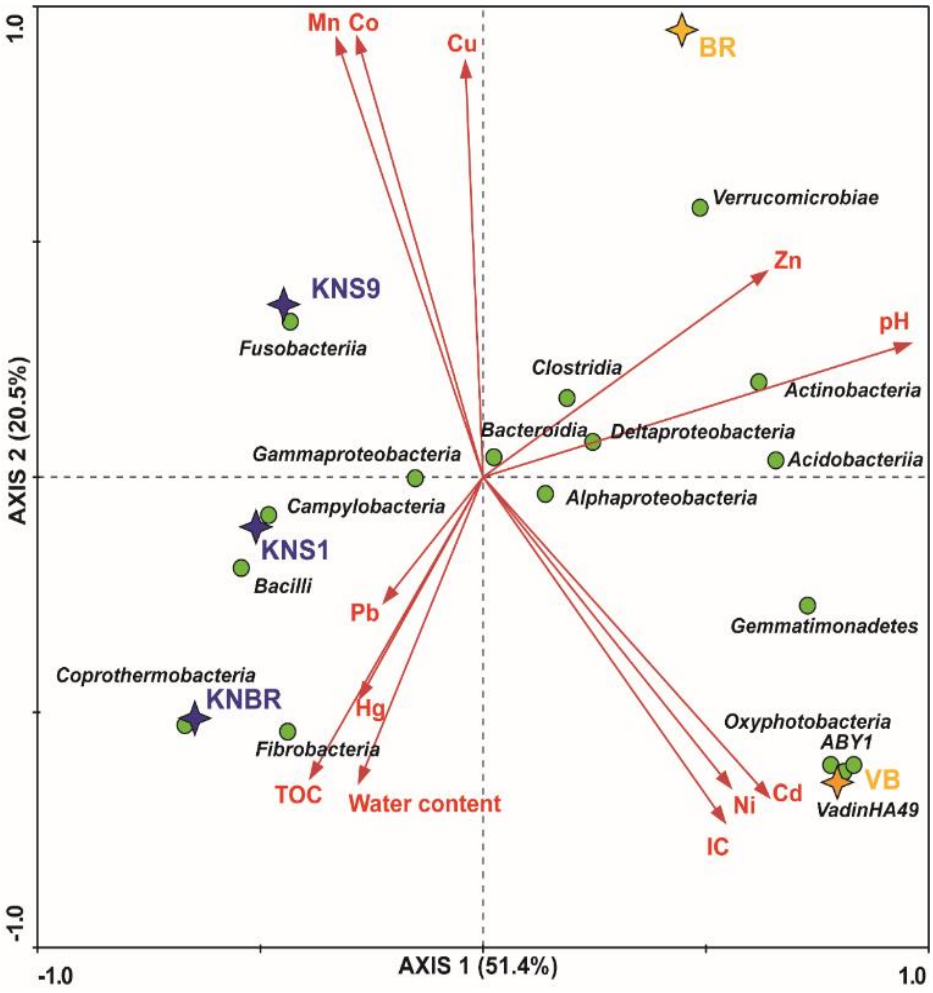

Fig. 3.15. Canonical correspondence analysis showing associations between the bacterial communities and geochemical properties of the terrestrial and fjord sediments. The red arrows represent environmental factors measured. The length of the arrows and the angle between each arrow and the nearest axis indicate the relative importance and closeness of that environmental factor in explaining the variation in the bacterial communities. The terrestrial sediments VB and BR are marked in orange stars and the fjord sediments KNBR, KNS1, and KNS9 are marked in blue stars.

Table 3.14.
Significant correlations between geochemical parameters, between geochemical parameters and bacterial classes, and between bacterial classes obtained by Pearson linear correlation.

Factors	r value obtained	Level of significance
Between bacterial class and geochemical parameters		
Acidobacteriia-porewater pH	0.878717	*
Actinobacteria-porewater pH	0.963051	**
ABY1-IC%	0.931711	*
Oxyphotobacteria-IC%	0.931711	*
Gemmatimonadetes-IC%	0.902067	*
vadinHA49-IC%	0.931711	*
Verrucomicrobiae-water content%	-0.93198	*
ABY1 (Patescibacteria)-Ni	0.921177	*
Oxyphotobacteria-Ni	0.921177	*
Gemmatimonadetes-Ni	0.892716	*
vadinHA49-Ni	0.921177	*
Clostridia-Zn	0.956256	*
Verrucomicrobiae-Zn	0.913656	*
Alphaproteobacteria-Cd	0.897676	*
Bacilli-Pb	0.931709	*
Bacilli-Hg	0.92968	*
Between bacterial classes		
Acidobacteriia-Gemmatimonadetes	0.902126	*
Acidobacteriia-Alphaproteobacteria	0.906927	*
Acidobacteriia-Deltaproteobacteria	0.961111	**
Actinobacteria-Gammaproteobacteria	-0.88154	*
Actinobacteria-Clostridia	0.92159	*
Coprothermobacteria-Fibrobacteria	0.999681	***
ABY1-Oxyphotobacteria	1	***
ABY1-Gemmatimonadetes	0.9966	***
ABY1-VadinHA49	1	***
ABY1-Alphaproteobacteria	0.912746	*
Oxyphotobacteria-Gemmatimonadetes	0.9966	***
Oxyphotobacteria-vadinHA49	1	***
Oxyphotobacteria-Alphaproteobacteria	0.912746	*
Campylobacteria-Bacilli	0.921514	*
Gemmatimonadetes-vadinHA49	0.9966	***
Gemmatimonadetes-Alphaproteobacteria	0.929046	*
Gemmatimonadetes-Deltaproteobacteria	0.901786	*
VadinHA49-Alphaproteobacteria	0.912746	*
Alphaproteobacteria-Deltaproteobacteria	`0.938896	*

*p value of 0.05 represented as *, p value of 0.01 (**) and p value of 0.001 (***)*

3(b). 3.5. Metabolic functional analysis of sediment communities and isolates

We could note significant differences in the community metabolic profiles between the terrestrial and fjord sediments at both 4 °C and 20 °C incubations ($p<0.005$) (**Fig. 3.16a and 16b**). However, at both temperatures, the terrestrial sediment community had shown a higher affinity to utilize amino acids, amines, and amides (24.8 - 35%) as compared to the fjord community (19 - 25.5%). Similarly, a higher affinity for utilization of carbohydrates and polymers was noted for the fjord sediment community irrespective of temperature incubations. It was found that all 5 sediment communities were positive for the utilization of D-mannitol, Tween 80, Tween 40, and N-acetyl-D-glucosamine (GlcNAc) at both 4 °C and 20 °C incubations (**Fig. 3.16a and 16b**). Polymer substrates such as α-cyclodextrin and glycogen and carbohydrate substrate D-cellobiose (product of cellulose degradation), were utilized by all 3 fjord sediment communities (OD > 0.5) at both temperature incubations (**Fig. 3.16a and 16b**). Similarly, Pyruvic acid methyl ester, D-glucosaminic acid, D-galactonic acid-gamma lactone, L-arginine, L-asparagine, and L-serine were utilized efficiently by the terrestrial sediment communities (OD > 0.5) over other substrates. Shannon-Wiener (H) index and Richness index (Rs) were higher for fjord sediments as compared to terrestrial sediments at 4 °C while the values were found to be lower than terrestrial sediments at 20 °C incubation (**Table 3.15**).

The total community metabolic response, however, might vary from the metabolic response of single bacteria representing each system. To test this hypothesis, the dominant cultivable bacterial fraction from both the terrestrial and fjord sediments was tested for its ability to grow in the presence of different carbon substrates. Some of the retrievable isolates chosen represented genera like *Flavobacterium, Polaromonas,* and *Photobacterium* which accounted for >5% relative abundance in the NGS results. For the experimental study, incubations at 4°C and 20°C were taken into account to determine the influence of varying temperatures on bacterial growth response. It was found that 50% of the terrestrial isolates and 70% of the fjord isolates could utilize at least 1 of the carbon substrates provided, at both temperature incubations (**Table 3.16**). Among the terrestrial isolates, the highest number of substrates were utilized by the

species *Cryobacterium roopkundense* and *Arthrobacter ginsengisoli* (100% substrates utilized at 4 °C and 42% at 20 °C) belonging to Actinobacteria and *Janthinobacterium lividum* (37% and 21% of substrates utilized at 4 °C and 20 °C) belonging to Proteobacteria. Similarly, among the fjord isolates, the maximum number of substrates were utilized by the species *Psychrobacter glaciei* (94.7% substrates utilized at 4 °C and 31.5% at 20 °C), *Photobacterium frigidiphilum* (47% at 4 °C and 26% at 20 °C), and *Psychrobacter nivimaris* (26% at 4 °C, 42% at 20 °C) belonging to Proteobacteria respectively (**Table 3.16**). PCA also showed that members of the bacterial phyla Proteobacteria and Actinobacteria have more affinity for utilization of different carbon substrates (**Fig. 3.17a and 17b**).

We have also looked into the metabolic profiles of the psychrophilic species isolated in our study. Among the isolates, *Polymorphobacter fuscus* utilized carbohydrate (NAG), polymer (Tween 80), and amino acid (L-serine) substrates at 4 °C while *Cryobacterium psychrotolerans* could utilize only carbohydrate substrates-D-mannitol and NAG at 4 °C. Similarly, *Polaromonas glacialis* could utilize only one amino acid substrate- L-serine at 4 °C. There was no substrate utilization observed at 20 °C except for the isolate *Polymorphobacter fuscus* which could utilize Tween80 at 20 °C. Therefore, among the three psychrophilic species, *Polymorphobacter* sp. had higher metabolic potential as compared to the other 2, although their metabolic capabilities were lower as compared to the psychrotolerant members isolated from the terrestrial system (**Table 3.16**).

We could also observe that at 4 °C incubation, maximum number of bacterial isolates have utilized N-acetyl-D-glucosamine > Sucrose > Tween 80 > D-mannitol = D-galacturonic acid = L-serine = L-glutamic acid > Cellulose = D-cellobiose. Similarly, at 20 °C, we found maximum utilization of Tween80 > D-mannitol > D-galacturonic acid > N-acetyl-D-glucosamine > D-cellobiose > D-glucose = D-lactose.

Similar to the community metabolic response, we could note significant differences in the bacterial growth yields (in terms of total cell count) towards different carbon substrates between the terrestrial and fjord isolates at both 4 °C and 20 °C incubations (p value < 0.05). **Fig. 3.18a** and **18b** indicate a decline in bacterial growth in terms of total cell count (*10^7cells/ml) towards different carbon sources at 20 °C incubation as

compared to 4 °C incubation. An exception was noted for the isolates F4 (*Planococcus halocryophilus*) and F7 (*Paeniglutamicibacter antarcticus*), wherein they yielded higher cell counts at 20 °C in the presence of different carbohydrates, polymers, and amino acid substrates.

Another aspect of our study was to check whether phylogenetically related bacterial members exhibit similar growth responses to varying substrates and varying temperature incubations or whether they are influenced by the sediment type. For this, isolates belonging to the species *Flavobacterium degerlachei*, one retrieved from the terrestrial sediment and one from the fjord sediment were included in the study. Similarly, different bacterial species belonging to the same genera were also considered for the experiment (*Cryobacterium roopkundense*, *Cryobacterium luteum*, and *Cryobacterium psychrotolerans* from the terrestrial sediments, *Psychrobacter glaciei, Psychrobacter fozii*, *Psychrobacter nivimaris*, and *Psychrobacter cryohalolentis* from the fjord sediments). Our results indicated a clear distinction in the growth response of different isolates irrespective of their phylogenetic relatedness (**Table 3.16**).

Table 3.15.

Community level physiological profiling results for each sediment sample incubated at 4 °C and 20 °C temperature incubations.

Sample	**AWCD**		**H**		**R**	
	4 °C	20 °C	4 °C	20 °C	4 °C	20 °C
VB	0.78	1.01	2.97	2.98	22	20
BR	0.46	1.43	2.83	3.13	16	26
KNBR	0.63	0.65	3.13	2.72	26	14
KNS1	0.69	1.04	3.06	3.07	24	22
KNS9	0.67	0.67	3.21	2.70	25	14

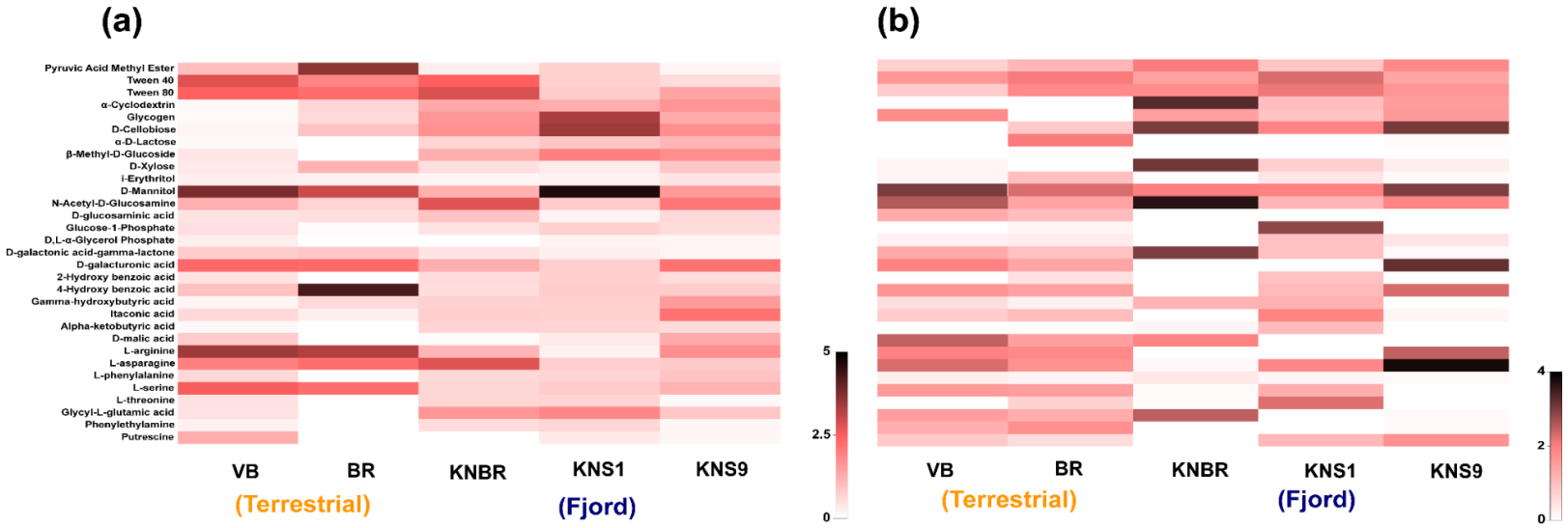

Fig. 3.16 (a) Heatmap of the carbon substrate utilization patterns on the Biolog Ecoplate for terrestrial and fjord sediments at 4 °C incubation and **(b)** Heatmap of the carbon substrate utilization patterns on the Biolog Ecoplate for terrestrial and fjord sediments at 20 °C incubation.

Table 3.16.

List of retrieved bacterial isolates selected for the experiment with their temperature range and number of substrates utilized for growth at 4 °C and 20 °C incubation.

Bacterial Isolates chosen	**Temperature optimum and range**	**No. of substrates utilized**	
TERRESTRIAL		4 °C	20 °C
Flavobacterium degerlachei (**4**VBSed**T**3)	15 - 20 °C (4 - 25 °C)	3	0
Arthrobacter ginsengisoli (**4**VBSed**A**2)	20 °C (4 - 35 °C)	19	8
Janthinobacterium lividum (**4**BR1Sed**T**2)	15 °C (4 - 30 °C)	7	4
Polymorphobacter fuscus (**20**BRSed**Z**4)	10 °C (4 - 15 °C)	3	1
Cryobacterium roopkundense (**4**VBSed**R**1)	20 °C (4 - 35 °C)	19	8
Cryobacterium luteum (**20**VBSed**R**2)	25 °C (4 - 30 °C)	2	0
Cryobacterium psychrotolerans (**4**BR2Sed**Z**2)	10 °C (4 - 15 °C)	2	0
Flavobacterium omnivorum (**20**VBSed**R**5)	15 °C (4 - 20 °C)	2	1
Polaromonas glacialis (**4**VBSed**T**8)	10 °C (4 - 20 °C)	1	0
Cellulomonas cellasea (**4**BR1Sed**T**3)	15 °C (10 - 20 °C)	0	0
FJORD			
Flavobacterium degerlachei (**4**KNS1Sed**Z**1)	15 °C (4 - 30 °C)	4	3
Psychrobacter glaciei (**4**KNS1Sed**A**4)	30 °C (4 - 35 °C)	18	6
Paracoccus sediminilitoris (**20**KNBRSed**Z**3)	10 °C (4 - 25 °C)	12	0
Planococcus halocryophilus (**4**KNS9Sed**Z**1)	25 °C (4 - 35 °C)	0	8
Psychrobacter fozii (**4**KNS1Sed**AA**3)	25 °C (4 - 35 °C)	4	3
Photobacterium frigidiphilum (**4**KNS9Sed**R**2)	25 °C (4 - 35 °C)	9	5
Paeniglutamicibacter antarcticus (**4**KNS9Sed**A**1)	20 °C (4 - 35 °C)	2	3
Psychrobacter nivimaris (**20**KNBRSed**R**2)	20 °C (4 - 35 °C)	5	8
Psychrobacter cryohalolentis (**4**KNS9Sed**A**2)	15 °C (4 - 20 °C)	0	0
Flavobacterium frigoris (**4**KNS1Sed**A**3)	15 °C (4 - 30 °C)	4	1

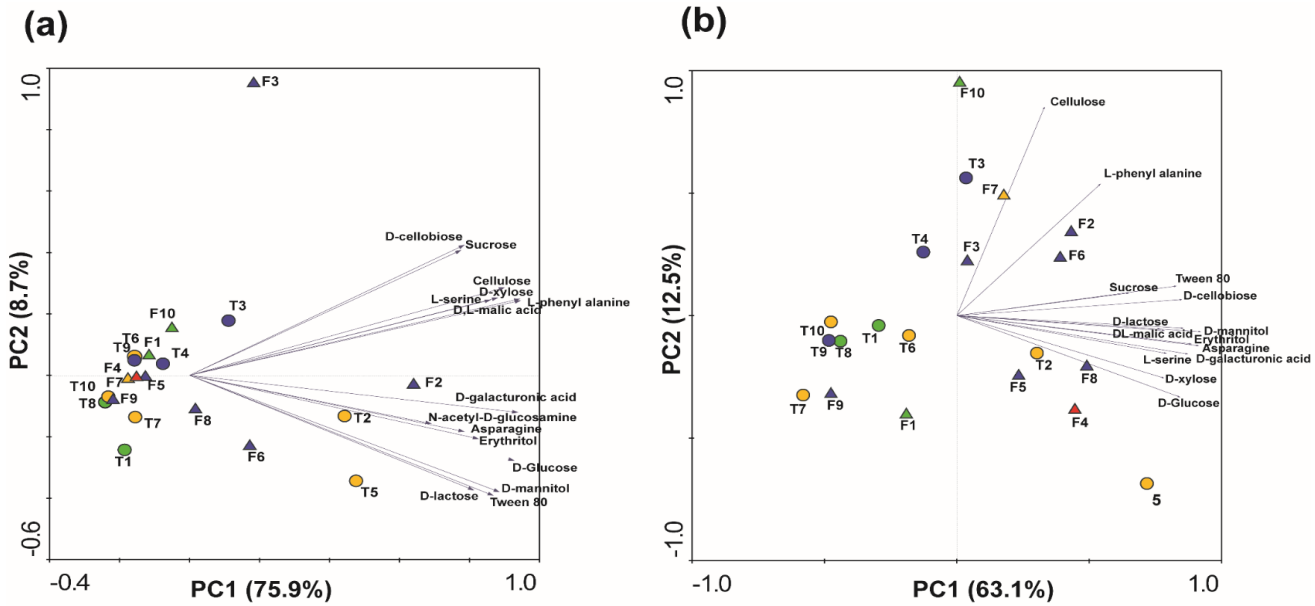

Fig. 3.17 (a) Principal component analysis (PCA) biplot of data showing scores and the variable vectors to ordinate bacterial isolates and the carbon sources which they can metabolize at 4 °C and **(b)** PCA depicting bacterial isolates and the carbon sources which they can metabolize at 20 °C temperature incubations. Percent variability explained by each principal component is shown in parentheses after each axis legend. Isolates in circle: Terrestrial isolates; Isolates in triangle: Fjord isolates. Different colours represent different bacterial phyla. Green-Bacteroidetes; Blue- Proteobacteria; Orange- Actinobacteria and Red- Firmicutes.
Details of the isolates: T1-*Flavobacterium degerlachei* (**4**VBSed**T**3), T2-*Arthrobacter ginsegisoli* (**4**VBSed**A**2), T3-*Janthinobacterium lividum* (**4**BR1Sed**T**2), T4- *Polymorphobacter fuscus* (**20**BRSed**Z**4), T5- *Cryobacterium roopkundense* (**4**VBSed**R**1), T6- *Cryobacterium luteum* (**20**VBSed**R**2), T7- *Cryobacterium psychrotolerans* (**4**BR2Sed**Z**2), T8- *Flavobacterium omnivorum* (**20**VBSed**R**5), T9- *Polaromonas glacialis* (**4**VBSed**T**8) and T10- *Cellulomonas cellasea* (**4**BR1Sed**T**3). F1- *Flavobacterium degerlachei* (**4**KNS1Sed**Z**1), F2- *Psychrobacter glaciei* (**4**KNS1Sed**A**4), F3- *Paracoccus sediminilitoris* (**20**KNBRSed**Z**3), F4- *Planococcus halocryophilus* (**4**KNS9Sed**Z**1), F5- *Psychrobacter fozii* (**4**KNS1Sed**AA**3), F6- *Photobacterium frigidiphilum* (**4**KNS9Sed**R**2), F7- *Paeniglutamicibacter antarcticus* (**4**KNS9Sed**A**1), F8- *Psychrobacter nivimaris* (**20**KNBRSed**R**2), F9- *Psychrobacter cryohalolentis* (**4**KNS9Sed**A**2), F10- *Flavobacterium frigoris* (**4**KNS1Sed**A**3).

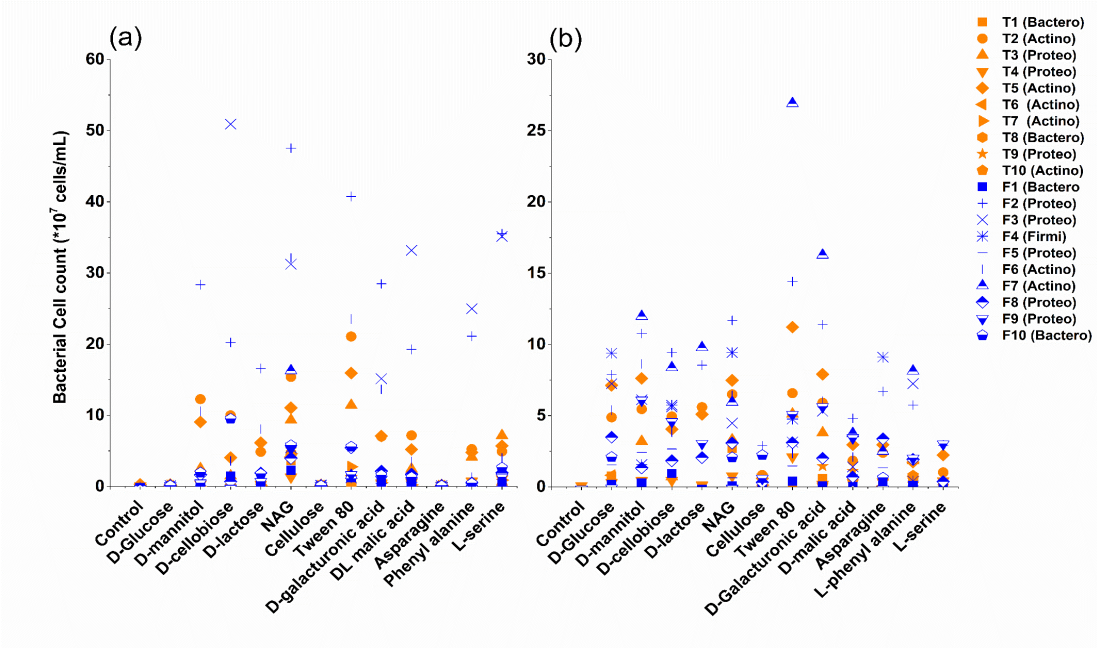

Fig. 3.18 (a) Bacterial growth yield results expressed in terms of bacterial cell count ($\times 10^7$cells/mL) for both terrestrial and fjord isolates grown in the presence of selected carbon substrates at 4 °C incubation observed at 12th day of growth. **(b)** Bacterial growth yield results for terrestrial and fjord isolates grown in the presence of selected carbon substrates at 20 °C incubation observed at 12th day of growth. Terrestrial isolates are marked as T1 - T10 while fjord isolates are marked as F1 - F10 along with the respective phylum details described in brackets as Bactero- Bacteroidetes, Actino-Actinobacteria, Proteo-Proteobacteria, and Firmi- Firmicutes.

3(b). 4. Discussion

The concentrations of the environmental factors such as TOC, IC, and trace elements varied between the terrestrial and fjord sediments in our study (**Table 3.9**). The highest TOC values observed in the outer fjord sediment (KNS1) with a reduction noted from outer to the inner fjord sediments corresponds to the previous reports (Kumar et al., 2016; Lu et al., 2013). High turbidity towards the fjord head (inner fjord) and steep gradient in sedimentation rate along with the supply of terrigenous carbonates and organic matter deprived glacier materials towards the fjord head may be critical in producing such a clear spatial gradient in TOC and IC values in the fjord sediments (Kumar et al., 2016; Koziorowska et al., 2017). Similarly, the results suggesting an increase in the concentration of Hg, Pb, Cd, and Cu from inner to outer fjord sediments is consistent with the notion that the Kongsfjorden waters are influenced by West Spitsbergen Current (WSC) which brings relatively warm and saline water as well as sediments from low and middle latitude to the Arctic (Lu et al., 2013). This in turn suggests the long-range transport of metals to the Arctic. The average values of trace metal concentrations of fjord sediments were comparable to the previously reported values from Kongsfjorden sediments (Lu et al., 2013; Grotti et al., 2017) and were lower than the baseline values reported by Lu et al. (2013) (baseline values for Ni: 26.22 mg/Kg, Co: 13.56 mg/Kg, Cu: 25.14 mg/Kg, Mn: 364.08 mg/Kg, Zn: 70.49 mg/Kg, Cd: 0.14 mg/Kg, Pb: 17.46 mg/Kg and Hg: 0.0098 mg/Kg). The exception was observed for the concentrations of Zn, Cd, and Hg, wherein the average concentrations were 1.9 times (Zn), 1.4 times (Cd), and 5 times (Hg) higher than the baseline values reported.

Similarly, while comparing the trace metal concentration of the terrestrial sediments with the baseline values reported from Ny-Ålesund soil samples by Hao et al. (2013), it was observed that the concentration of Ni, Cu, and Zn were higher in our study (baseline values reported for Ny-Ålesund soil- Ni: 6.24 mg/Kg, Cu: 11.5 mg/Kg, Zn: 77.3 mg/Kg, Pb: 4.38 mg/Kg, and Hg: 0.27 mg/Kg). High concentrations of Zn and Cu were reported from the cryoconite samples from Midtre lovenbreen glacier; Zn: 132 - 150 mg/Kg and Cu: 39 - 44 mg/Kg (Singh et al., 2013) and from Hans glacier; Zn: 84

- 108 mg/Kg and Cu: 23 - 49 mg/Kg (Łokas et al., 2016) which were comparable to our VB and BR sediment trace metal values. The role of atmospheric circulation transporting metals from global and local sources, marine aerosols containing metals, precipitation, and the abundance of soluble rocks such as carbonates and sulphides, and the contact of water with glacial debris act as the main suppliers of trace metals in the Arctic glacier associated and terrestrial environments (Łokas et al., 2016; Dragon and Marciniak, 2010). The significant correlation of TOC-Hg observed in our study was suggested by Lu et al. (2013) and may indicate the possible association of Hg to organic materials in the fjord sediments (Grotti et al., 2017). Similarly, the association of Hg-Pb and Mn - Co is an indication that these metals had a similar source or may be produced by similar physicochemical processes (Bai et al., 2011).

The total microbial count observed in the terrestrial sediments was two-fold lower as compared to the counts from the fjord sediments in this study. Reddy et al. (2009) has reported a total bacterial count in the range of 2.7 - 11 × 10^7 cells/g for the sediment samples collected from the snout of Midtre Lov´enbreen glacier to the confluence point of the meltwater streams of the glacier with the fjord. Similarly, Conte et al. (2018) has reported the total microbial counts from the Kongsfjorden sediments to be in the range of 10^7 cells/g which is one - fold lower than the values observed in our study.

Pigmentation observed for more than half of the total terrestrial and fjord sediment retrievable bacterial fraction has been reported as an adaptive strategy to cold environments and for UV protection (Mueller et al., 2005). The higher abundance of bacterial isolates affiliated to the phylum Actinobacteria in the terrestrial sediments corroborated well with the studies by Edwards et al. (2013) and Zhang et al. (2016) wherein they indicated the presence of Actinobacterial members as common inhabitants of glacier forelands and cryoconites, playing a significant role in soil development and biogeochemical cycling. The three psychrophilic bacterial species isolated in our study from the terrestrial sediments are known representatives of glacier systems of Polar and high elevation environments (Zhang et al., 2007; Margesin et al., 2012; Jia et al., 2015). Of which, the genus *Cryobacterium* has been previously reported as a dominant genus associated with the supraglacial systems, known to decompose a wide variety of organic carbon substrates (Poniecka et al., 2020). Liu et

al. (2020) has also observed a significantly higher number of genes involved in stress response, motility, and chemotaxis in the genus *Cryobacterium* as cold adaptive survival strategies. Similar properties of carbon substrate utilization and mechanisms to withstand unfavourable conditions - through dormancy (Darcy et al., 2011) and plasmid-mediated genes (Ciok et al., 2018) have been reported in the genus *Polaromonas.* The third psychrophilic species belonged to the genus *Polymorphobacter*. *Polymorphobacter* genus comprises all psychrotolerant members isolated from low-temperature environments and known for their aerobic anoxygenic photoheterotrophic nature (Jia et al., 2015; Phurbu et al., 2020). Other dominant terrestrial isolates belonged to *Janthinobacterium* sp. which are known to produce water-insoluble purple pigment violacein as environmental stress responsive mechanism (Pantanella et al., 2007) and *Flavobacterium* sp. known for organic matter degradation in the glacier environment (Poniecka et al., 2020; Thomas et al., 2020).

Higher abundance of γ-proteobacterial genus *Psychrobacte*r in the fjord sediments (**Table 3.12a and 12b)** corroborates with the previous culture-based studies from Kongsfjorden where their potential role in carbohydrate degradation, as well as cold-active enzyme production, was demonstrated (Prasad et al., 2014; Jain and Krishnan, 2017a). The retrieval of Actinobacterial species only from the inner fjord sediments might suggest the association of Actinobacterial taxa with the cold and less saline environment.

Flavobacterium degerlachei, belonging to the phylum Bacteroidetes, was the only common representative retrieved from both the terrestrial and fjord sediments from both incubation temperatures (**Table 3.11**) suggesting their adaptability to both the terrestrial and fjord environments and corresponds well with our previous report from the meltwaters and fjord waters (Thomas et al., 2020). The versatile nature of the *Flavobacterium* species could be linked to their ability to produce poly-unsaturated fatty acids, cold-adapted enzymes, and pigments along with their specialized role in uptake and degradation of high molecular weight fraction of organic matter (Liu et al., 2019).

A drop in biodiversity from the glacier snout to the downstream fjord sediments was observed which is in line with our previous results from glacio-marine waters (Thomas

et al., 2020). The higher Shannon diversity values for the terrestrial sediments are comparable to the values reported from soils and sediments from an Arctic lake in Svalbard (Wang et al., 2016). Despite the lower temperature and oligotrophic conditions prevailing in the glacier environment, the higher diversity noted could be maintained by the mechanism of transient dormancy of the microbiota (Jones and Lennon, 2010). The Shannon diversity values for fjord sediments are in agreement with those reported by Zeng et al. (2017).

The dominance of sequences affiliated to Proteobacteria, Bacteroidetes, and Verrucomicrobia from VB and BR sediments were previously reported for Svalbard lake sediments (Wang et al., 2016), although a comparatively lesser abundance of Acidobacterial phylum was found in the present study. The abundance and exclusive presence of cyanobacterial OTUs in the snout sediment (VB) might indicate their export from the supra-glacier systems to the snout region as cyanobacterial members are known to dominate the supraglacial ecosystems such as cryoconites playing a significant role in microbial community interactions and involved in cryoconite granule formation (Anesio et al. 2017; Garcia-Lopez and Cid 2017). The dominance of Verrucomicrobial OTUs in the forefield sediment BR is consistent with the results of Bendia et al. (2018), wherein they suggested the role of Verrucomicrobia in methane cycling in the glacier environments. Significant correlations were observed (**Table 3.14**) between the bacterial classes Oxyphotobacteria (Cyanobacteria), VadinHA49 (Planctomycetes), ABY1 (Patescibacteria), and Alphaproteobacteria, which are dominant in terrestrial sediments suggesting their possible interactions with each other in the terrestrial system, contributing to ecosystem functioning. Such links were reported by Kosek et al. (2019) from Revelva catchment waters, wherein the abundance of Planctomycetes and Alphaproteobacteria in areas covered by cyanobacterial mats was related to their role in the degradation of algal matter. Further, the glacier snout sediment VB harbored highly diverse rare taxa (bacterial taxa having abundance < 0.1%) as compared to the other samples (64.8% of total rare taxa) (**Fig. 3.14**), which in turn points out the importance of snout ecosystem acting as a 'seed bank' with less abundant highly diverse dormant taxa having unknown functional potential that could become numerically dominant and active with favorable environmental conditions

(Dawson et al., 2017). This observation was further supported by the presence of 5 potential novel bacterial species from the retrievable fraction from the VB sediment (**Table 3.11**) also indicating the snout sediment to be a hotspot of rare taxa.

The higher abundance of Flavobacterial OTUs (12% of the total) in VB sediment, suggests their possible role in algal matter degradation, as supported by the presence of algal mats in the snout region. The cyanobacterial genus *Phormidesmis* observed exclusively in the VB sediment is a known cyanobacterial taxon from Polar regions involved in the release of an extracellular polymeric substance (EPS) and the development of cryoconite granules (Anesio et al., 2017). Similarly, the presence of chemolithotrophic Fe^{3+} reducing bacteria belonging to the genus *Rhodoferax* from the Svalbard coastal and tidewater glaciers was reported by Garcia-Lopez et al. (2019). Psychrotolerant representatives of the genus *Luteolibacter* have been isolated and characterized from the tundra soil ecosystems from Svalbard (Kim et al., 2015) while members of the genus *Polaromonas* have been recognized as a significant component of glacial microbiomes, known for accelerating mineral weathering in glacier forelands (Ciok et al., 2018; Frey et al., 2010). The genus *Clostridium* observed as abundant taxa from the glacier forefield sediment, BR is known to act as precursors for sulfate reducers and methanogens by the production of organic acids and hence performing an important step in anaerobic decomposition of organic matter in aquatic ecosystems (Wang et al., 2016). Also, the presence of the *Clostridium* genus as key fecal indicators in the Revelva catchment water samples has been reported by Kosek et al. (2019), wherein they suggest the role of animals and birds in supplying nutrients throughout the catchment with fecal matter. The sequences belonging to the genus *Polaromonas* and *Geobacter* as a core group of diazotrophs were reported from Arctic glacier-forefields (Nash et al., 2018). These taxa were observed exclusively in the terrestrial sediments in our study, suggesting their niche-specific role in nitrogen fixation in glacier sediments. The culture-dependent and culture-independent analysis of the snout and forefield sediments indicated that their community comprise of both cosmopolitan taxa reported from the overlying meltwaters (Thomas et al., 2020), the supra- and subglacial systems (Stibal et al., 2012; Poniecka et al., 2020), and glacier-forefield soils (Nash et al., 2018; Malard et al., 2019) and rare taxa specific to the sediments.

The dominance of Proteobacteria (γ-proteobacteria), Epsilonbacteraeota, and Bacteroidetes observed in the Kongsfjorden sediments corresponds to the results of Zeng et al. (2017). An increased abundance of OTUs belonging to Epsilonbacteraeota was noted from the inner to the outer fjord sediment (**Fig. 3.13**). This could be due to the influence of Atlantic water (Hamdan et al., 2013), which is more prevalent in the outer fjord (Cottier et al., 2005). The genera *Sulfurimonas* and *Sulfurovum* belonging to Epsilonbacteraeota represented a substantial portion of the fjord sediment microbiome which corresponds to the previous studies from Kongsfjorden sediments, suggesting their role in sulfur cycling in marine environments (Zeng et al., 2017; Campbell et al., 2006). The dominance of Fusobacterial OTUs with the dominant genus being *Psychrilyobacter* was detected exclusively in the inner fjord sediment (KNS9). The presence of *Psychrilyobacter* species as key players in complex organic matter degradation, associated with the death and deposition of primary producers such as cyanobacteria in the Arctic marine sediments was noted by Müller et al. (2018). A similar observation was noted by Canion et al. (2014) wherein, an experimental treatment of Arctic fjord sediments with diatom-derived organic matter resulted in an increase in OTUs belonging to γ-proteobacteria (*Photobacterium* and *Colwellia*) and Fusobacteria (*Psychrilyobacter*). The predominance of the genus *Photobacterium* belonging to the order *Vibrionales* (γ-proteobacteria) in all 3 fjord sediments in our study, could be supported by their global distribution in marine sediments and for their known ability for cold-adapted enzyme production (Wang et al., 2019). The *Photobacterium* genus accounted for the vast majority of sequences associated with the gut of migrating Atlantic cod (Le Doujet et al., 2019), which in turn is a common fish in deep waters in Kongsfjorden contributing majorly to the total fish biomass in the fjord (Hop et al., 2002). This could imply the introduction of these bacteria through fecal pellets or dead matter from fishes into the fjord sediments, with a major role in breaking down biomass containing fibrous proteins, carbohydrates, and other macromolecules (Le Doujet et al., 2019). Increased recovery of *Flavobacterial* OTUs from the outer and inner fjord sediments suggests their key role in the assimilation of phytodetritus in the sediments and could represent one of the key taxa involved in complex polysaccharide degradation in the sediments (Teske et al., 2011). The

dominance of OTUs belonging to class γ-proteobacteria (30.5 - 44.5%) and Bacteroidia (7.5 - 20.6%) in all fjord sediments is substantiated by our retrievable data, suggesting a vital linkage of the dominant bacterial taxa in the fjord sediments with organic matter degradation (Zeng et al. 2013).

While comparing the amplicon sequencing results with the cultivable isolates, we could observe that all the species isolated in our study had their closest sequence variants among the OTUs from the amplicon results. Although cultivation-dependent techniques have the limitation of the selectivity of isolation media and culture conditions, it still stands significant in studying the physiological and metabolic potential of bacterial isolates from cold Polar environments which are otherwise not possible with the 16SrRNA amplicon sequencing approach. The combination of both these approaches has clearly described differences between the terrestrial and fjord sediments in terms of their community structure.

The significant influence of pH on the bacterial community structuring and their significant correlation with Acidobacterial and Actinobacterial OTUs (**Table 3.14**) was comparable to the results from the pan-Arctic survey of bacterial communities in Arctic soils by Malard et al. (2019). Similar correlations were noted for Ni, Cd, and Inorganic Carbon (IC) with terrestrial bacterial taxa. Likewise, the close association of Pb, Hg, TOC, and water content with the fjord sediment communities was noted (**Fig. 3.15**). Therefore, our results establish a linkage between the environmental factors and bacterial community structuring associated with the terrestrial and fjord system.

Our culture-dependent and culture-independent bacterial diversity analysis indicated that the dominant members of the terrestrial and fjord sediments are known for their capability for organic carbon degradation. The community level metabolic profiles of the terrestrial and fjord sediments differed significantly at both 4 °C and 20 °C incubations ($p < 0.005$) which might be due to the influence of environmental factors or the substrate availability in each of the systems. We could also observe the utilization of substrates such as D-mannitol, Tween 80, Tween 40, and N-acetyl-D-glucosamine (GlcNAc) at both 4 °C and 20 °C incubations by both terrestrial and fjord communities (**Fig. 3.16 (a) and (b)**). Mannitol being low - molecular-mass organic osmolytes, are accumulated in the cytoplasm by cold-adapted bacteria, to lower the cytoplasmic

freezing point and avoid desiccation by counteracting water loss and cell shrinkage during freezing (Collins and Margesin, 2019). Feltracco et al. (2020) reported the prevalence of alcohol sugar mannitol in coarse particles in Arctic aerosol denoting it as a local biogenic source. GlcNAc is a low-molecular-weight amino sugar that is used for the synthesis of cell surface structures in bacteria and plays an important role in supplying carbon and energy by entering the glycolytic pathway after it is converted into fructose-6-phosphate (Álvarez-Añorve et al. 2005). GlcNAc uptake is reported as a widespread phenotype among marine bacteria by Cottrell and Kirchman (2000)**.** Similarly, the Tween compounds, having an oleic acid moiety in their structure are commonly used by polar bacteria (Sala et al., 2008) and known to protect bacterial cells from adverse environmental conditions (Reitermayer et al., 2018). This is an indication that the terrestrial and fjord sediment communities utilize certain specific carbon substrates which could boost their survival mechanisms in Arctic environments.

Higher affinity for carbohydrates and complex polymer substrates over others observed in the fjord sediments suggests the presence of complex carbohydrate degrading bacterial groups in the fjord sediments (Teske et al., 2011; Jain and Krishnan, 2017a). Shannon-Wiener (H) index and Richness index (Rs) were higher for fjord sediments as compared to terrestrial sediments at 4 °C while the values were found to be lower than terrestrial sediments at 20 °C incubation (**Table 3.15**). At these two temperatures, terrestrial and fjord communities showed variation in their metabolic responses, which may have significant implications on the substrate availability in the natural environment.

The metabolic response of single bacterial isolates was also considered by testing the dominant cultivable bacterial fraction from both the terrestrial and fjord sediments for their affinity to utilize different carbon substrates. This yielded significant differences in the bacterial growth yields towards different carbon substrates between the terrestrial and fjord isolates at both 4 °C and 20 °C incubations ($p < 0.05$). Comparison between the psychrophilic and psychrotolerant representatives of the genus *Cryobacterium* (*C. psychrotolerans, C. roopkundense, C. luteum*) indicated distinct metabolic capabilities for different species belonging to the same genera isolated from the same environment.

The utilization of N-acetyl-D-glucosamine, D-mannitol, and Tween 80 by the terrestrial and fjord isolates irrespective of 4 °C and 20 °C incubations corroborate well with the Ecoplate results suggesting the ecological importance of these substrates in the Arctic sedimentary environments. There was a noticeable temperature selection, particularly for the terrestrial isolates i.e. 4 °C incubation found to have higher growth yields for different substrates than 20 °C incubation. Production of cold-adapted enzymes having a high level of specific activity at low temperatures might have enhanced their affinity to utilize different substrates at a lower temperature (Struvay and Feller 2012). This needs further evidence through experiments involving varying temperatures and varying substrate concentrations as well as whole-genome-based studies on the psychrotolerant isolates which can shed light on the different mechanisms involved in high metabolic activity at temperatures much below their temperature optima for growth.

Our study also indicated a clear distinction in the growth response of isolates belonging to the

- same species - *Flavobacterium degerlachei,* one retrieved from the terrestrial sediment and one from the fjord sediment
- same genera - *Cryobacterium roopkundense, Cryobacterium luteum,* and *Cryobacterium psychrotolerans* from the terrestrial sediments, *Psychrobacter glaciei, Psychrobacter fozii*, *Psychrobacter nivimaris* and *Psychrobacter cryohalolentis* from the fjord sediments (Thomas et al., 2021)

Similar results were reported by Poniecka et al. (2020) wherein they observed striking differences in the metabolic capabilities of closely related isolates assigned to the same OTU. Since bacterial strains/species can adjust their substrate utilization profiles according to condition changes, the phylogenetic origin seems to be a less important structuring component of sediment bacterial communities (Freese et al. 2010). Detailed whole-genome level studies could further give insights into the metabolic pathways in phylogenetically similar bacterial species from terrestrial and fjord environments.

Therefore, our study suggests the adaptive responses of bacterial members to utilize the available substrates in the varying sedimentary environment. Utilization of D-

mannitol, N-acetyl-D-glucosamine, and Tween 80 by both terrestrial and fjord sediment communities as well as by the cultivated bacterial fraction indicates the readily available nature of such carbon substrates in the Arctic sedimentary environments, promoting survivability of bacteria in the extremely cold environments (Thomas et al., 2021). The significant influence of temperature on the growth response of terrestrial and fjord isolates towards different carbon substrates was also indicated in our study, with phylogeny found to be a less important structuring component of bacterial metabolic potential.

Chapter 4.

Insights into metal tolerant bacterial diversity associated with a glacio-marine system in Ny-Ålesund, Arctic

Chapter. 4.

Insights into metal tolerant bacterial diversity associated with a glacio-marine system in Ny-Ålesund, Arctic

4.1. Introduction

Over the last few decades there are increased reports on the introduction of contaminants into the Arctic via long-range transport from atmospheric and oceanic circulation, localized anthropogenic activities such as metal mining, shipping, oil exploration, military, and tourism activities, etc (Mortazavi et al., 2019; Amuno et al., 2016, Trefry et al., 2013; Riedel, 2014; Strand et al., 2002). Also, climate warming and the resulting glacier retreat and sea-ice melting are causing an increased release of contaminants in the Arctic (Bogdal et al., 2010). In particular, the seasonal daylight conditions and extended periods of darkness during winter, low average annual temperature, ice, and snow favor the potential accumulation of metals in both biotic and abiotic matrices (Hung et al., 2010).

Bazzano et al. (2014) has reported the different sources for metals in Arctic fjords and suggested glaciers as the major source for Co, and Fe and anthropogenic influence to be the major source for Cu, Mn, Mo, Ni, Pb and Zn which are introduced through the intrusion of Atlantic waters. The persistent nature of heavy metals eases them to enter the food web, which further can have detrimental impacts on the ecosystem (Dietz et al., 2000). Biomagnification of heavy metals through different trophic levels in the food chain means that even low background levels of metals and low levels of atmospheric deposition may produce a powerful multiplier effect, resulting in significant heavy metal concentrations in higher Arctic organisms (Dietz et al., 2000; McConnell and Edwards, 2008).

Higher concentrations of heavy metals highly influence the microbial communities by inhibiting metabolic functions, altering enzymes specificity, and even leading to a shift in microbial community structure. Heavy metals could also damage the cell

membranes, disrupt cellular functions, denature DNA and proteins, and may also interfere with oxidative phosphorylation and osmotic balance (Zhang et al., 2016; Nath et al., 2019). Microbial responses to heavy metals provide a significant model for ecological studies to assess the environmental impact on biodiversity (Guo et al., 2009) and how they effectively play role in metal mitigation. Bacteria play a significant role in the fate of metal pollutants in the environment by either causing volatilization, precipitation, or complexation of the metals (Gillan et al., 2005), by efflux mechanisms, intracellular and extracellular sequestration of metals or by enzymatic reduction of metal ions (Ianieva, 2009). Some bacteria could even transform metals into more toxic organic forms (eg. Methylated derivatives) which further enter the food chain and impact higher trophic levels (Duan et al., 2020; Ianieva, 2009).

Although metal resistance of bacteria from environments such as wastewater, sewage, and polluted soils are studied extensively, studies on metal tolerant bacteria from Polar Regions especially Svalbard, Arctic are limited (Rappazzo et al., 2019; Caputo et al., 2019; Balan et al., 2018; Neethu et al., 2015). Svalbard archipelago in the Arctic is known to be highly sensitive to climatic impacts and there are many reports on the increased metal concentrations in the West Spitsbergen fjords of Svalbard and the associated terrestrial regions (Kalinowska et al., 2020; Grotti et al., 2017; Bazzano et al., 2014; Halbach et al., 2017; Hao et al., 2013). This suggests the importance of this region for metal tolerant bacterial studies. Caputo et al. (2019) studied the metal resistance in bacteria from contaminated Pasvik river sediments in the Arctic and indicated a linkage between the bacterial metal resistance and an increase/decrease of metal concentrations that influenced bacterial distribution in the region. Similarly, Neethu et al. (2015) suggested the association of antibiotic resistance with metal tolerance in heavy metal resistant gram-negative bacteria isolated from Kongsfjorden water and sediment samples. These studies have provided an impetus to investigate further whether the metal tolerant bacterial fraction in an Arctic glacio-marine system is influenced by the type of metals or by the sampling location or type of samples.

Therefore, this chapter focuses on the metal tolerant bacterial fraction associated with the Vestrebroggerbreen glacier, its foreland meltwater channel system, and the downstream fjord system in the Svalbard, Arctic using enrichments with metals such

as Hg, Pb, Cd, Ni, Co, Zn and Mn (both toxic and essential category of metals). Also, the induction of other resistant properties such as antibiotic resistance in selected metal tolerant Arctic bacterial isolates was investigated in this chapter.

4.2. Materials and Methods

4.2.1. Sample details

The samples used for the study included ice (VB ice) and sediments from VestreBroggerbreen (VB) glacier snout region, meltwaters and sediment samples from the glacier foreland region (BR), subsurface water samples, and surface sediments collected from the outer, middle, and inner regions of Kongsfjorden (**Fig. 4.1**). The samples were collected during the Indian Arctic expedition 2017 (August-September). A total of 3 sediment samples collected from the Vestrebroggerbreen glacier snout region (VB1 to VB3) were pooled together to form a single representative sample VBsed. Similarly, 3 sediment samples were collected from the glacier foreland region (BR2, BR3, and BR4) and pooled together to form a homogenous single sample BRsed.

4.2.2. Trace metal measurements

The background trace metal concentrations were measured using the Inductively-coupled Plasma Mass Spectrometer (Thermo iCAP Q ICP-MS, Thermo Fisher Scientific, USA). The procedure for water sample analysis is detailed in **Chapter. 3. Section 3(a). 2.3** and the sample preparation and digestion protocol for sediment sample trace metal measurements is described in **Chapter. 3. Section 3(b). 2.2.**

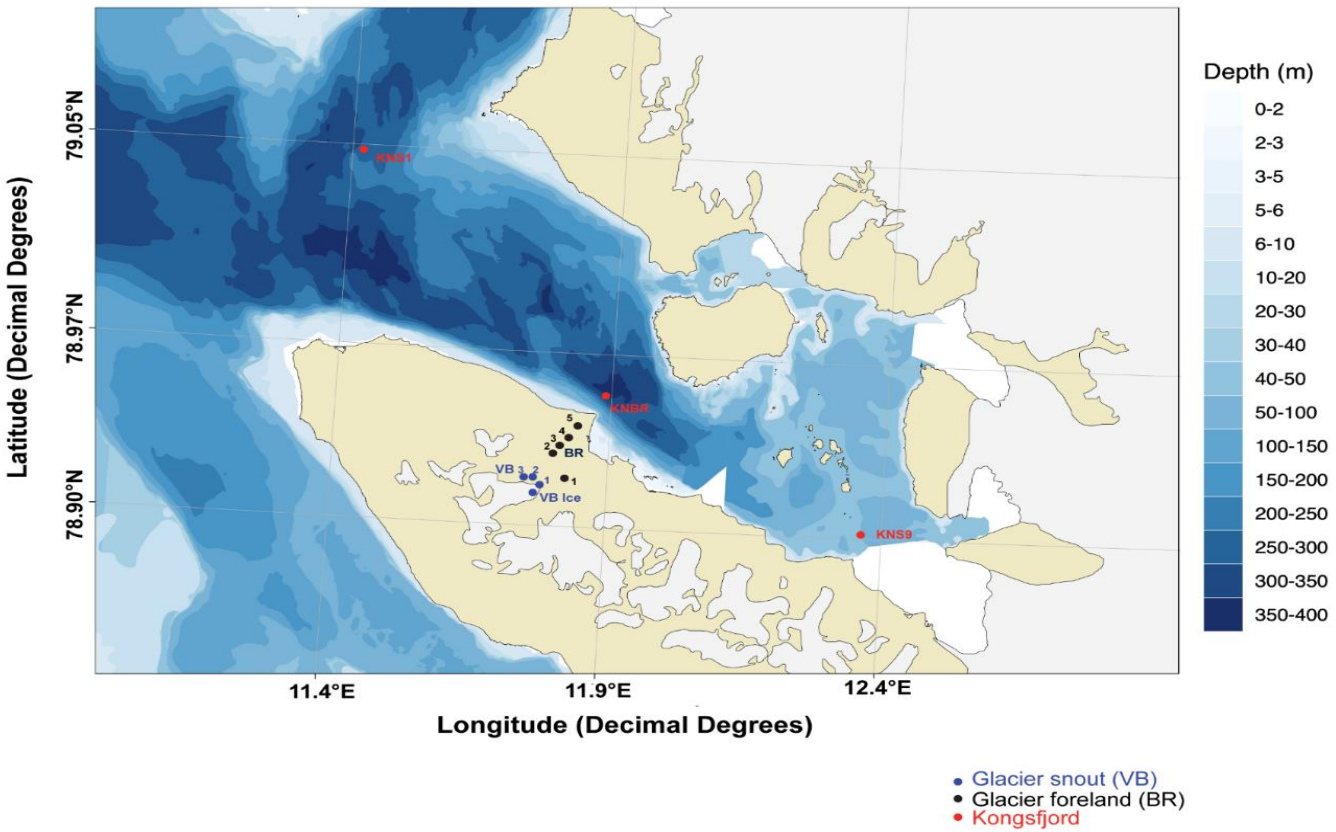

Fig. 4.1. Map of the glacio-marine sampling stations. VB 1, 2, and 3 samples pooled together to obtain VBsed. BR1, BR2, BR3, BR4, and BR5 represent the meltwater samples collected from the foreland region of the Vestrebroggerbreen glacier. Sediment samples were collected from locations BR2, BR3, and BR4 and pooled together to form a single representative sediment sample, BRsed. KNS1, KNBR, and KNS9 represent the outer, middle, and inner fjord locations from which water and surface sediment samples were collected.

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4.2.3. Enrichment experiment

For the enrichment experiment, samples were suspended in a low concentration Nutrient broth media ($1/100^{th}$ strength, M002, HiMedia) referred to as Minimal media (MM) with different metal amendments. The metals used for the study included Hg, Cd, Pb, Ni, Co, Zn, and Mn in the form of their analytical grade salts - $HgCl_2$, $CdCl_2.H_2O$, $ZnCl_2$, $Pb(NO_3)_2$, $NiCl_2.6H_2O$, $CoCl_2.6H_2O$, and $MnCl_2.4H_2O$ respectively (Merck). For the metal enrichments, Hg (5µM), Cd (50µM), Pb (50µM), Ni (200µM), Co (200µM), Zn (250µM), Mn (2.5mM) along with combinations of metals C1 (Hg (2µM), Pb and Cd (10µM)) and C2 (100µM each of Ni, Co, Zn, and Mn) were used. The above metals and their concentrations were chosen based on a pilot study on fjord sediments using different metal amendments performed in the lab at NCPOR and also by assessing the baseline values of metals reported from the Kongsfjorden sediments by Lu et al. (2013). For the melted ice sample and water samples from glacier foreland and fjord, 250µL of samples were inoculated into 25 ml of MM with metals for enrichment. For the sediment samples, the sample suspension was prepared by sonicating 5g of sediment in 45mL of filter-sterilized saline (0.9%) before the enrichment (250µL of sonicated sediment samples into 25 ml of MM with metals). Metal-amended samples in MM were incubated at 15°C for three weeks followed by the isolation of metal tolerant isolates by spread plating onto MM Agar plates with respective metals. Morphologically distinct colonies thus obtained were purified onto metal amended plates.

4.2.4. DNA extraction, 16SrRNA gene sequencing, and taxonomic identification

Total bacterial genomic DNA extraction from pure cultures, rep-PCR screening using BOX A1R primer, and 16S rRNA gene amplification, and sequencing were carried out as detailed in **Chapter 3. Section 3(a).2.5**. The trimming and sequence assembly for the obtained sequences were carried out using Codon code Aligner software Version 8.0.2 and the obtained consensus contigs were screened for chimera sequences using USEARCH version 6.0 (http://rdp8.cme.msu.edu/cgis/chimera.cgi) and were removed

from the analysis. The 16S rRNA gene nucleotide sequences having greater than 1000 bp were further analyzed in the EZbiocloud server (Yoon et al., 2017).

4.2.5. Screening for metal tolerant genetic elements and sequencing

The details of the primer pairs used for the detection of various metal tolerant genes are given in **Table 4.1**. For each PCR reaction, the DNA from all identified bacterial strains was used as a matrix (1 µL for 25µL reaction volume). PCR was performed for a total reaction volume of 25µL, in a SimpliAmp Thermal cycler (Thermofisher Scientific, USA), using HiThroughput EmeraldAmp GT PCR mastermix (Takara, Japan) (12.5 µL) and appropriate primer pairs (0.3pM/µL of primer). The optimal PCR conditions were as follows: initial denaturation at 94°C-95°C for 5 min followed by 29 cycles of 30 sec at 94°C, 40-45 sec at optimal annealing temperatures (**Table 4.1**) and 1 min at 72°C, and a final extension of 10 min at 72°C. The amplified PCR products were purified using the ExoSAP-IT PCR product cleanup reagent (Thermofisher Scientific, USA) followed by sequencing using Applied Biosystems 3500 Genetic analyser (Thermofisher Scientific, USA). The trimming and sequence assembly for the obtained sequences were carried out as discussed in the previous section. The metal gene nucleotide sequences were analyzed on the NCBI-Blastn server.

4.2.6. Phylogenetic analysis

Phylogenetic analyses for the 16SrRNA gene sequences of selected isolates and metal-specific gene sequences were conducted using MEGA7 (Kumar et al., 2016), applying the Neighbor-Joining (NJ) algorithm (with the Kimura-2 with gamma distribution model) and 1000 bootstrap replicates. Sequence alignments were prepared using the ClustalW algorithm (Chenna et al. 2003).

4.2.7. Secondary screening of bacterial cultures in metal-containing broth media

The 98 identified metal tolerant isolates obtained from the metal amended plates were further screened for their tolerance to different metal concentrations in MM (Hg (5, 7.5, and 10µM), Cd (50, 75, and 100µM), Pb (50, 75, and 100µM), Ni and Co (200 and 500µM), Zn (250 and 500µM), and Mn (2.5, 5, and 10mM)) and their multi-metal

tolerance potentials. 20 isolates out of the total were shortlisted based on their multi-metal tolerance potential and further subjected for Minimum Inhibitory Concentration (MIC) determination.

For MIC determination, MM with metal concentrations was prepared in 30 mL acid-washed test tubes (2% Supra-Pure Nitric acid, Merck), with a final broth volume of 15 mL in each tube. The metal concentrations used were Hg- 5, 10, 25, 50 µM, Cd and Pb- 10, 25, 50, 100, 150, 250, 500 µM, Ni, Co and Zn- 100, 150, 250, 500, 750, 1000, 2500 µM, and Mn- 250, 500, 750, 1000, 2500, 5000, 10000, 25000, 50000 µM. Into each tube, 150 µL of actively growing culture was added (OD value of 0.5 at 600 nm) and corresponding cell counts were estimated using DAPI fixing and epifluorescence microscopy (Chapter 3. Section 3(a). 2.4).

A tube without metal was taken as positive control and another tube without the culture served as the negative control. Tubes were incubated at 15°C for 15 days. All tests were conducted in triplicates. The tube with the lowest concentration of the metal that inhibited growth was taken as the MIC of the metal.

Table. 4.1.
Primers used to target metal resistance genes, their sequence, functions, expected length, and annealing temperatures.

Primer name	Sequence	Function	Length	Annealing temp	Reference
merAHF	5′-CCA TCG GCG GCA CYT GCG TYAA-3’	Mercuric reductase	1247bp	66°C	Wang et al., 2011
merAHR	5′-CGC YGC RAG CTT YAA YCY YTC RRC CAT YGT -3′				
merALF	5′-CGTSAACGTSGGSTGC GTG CCSTCC AAG -3′	Mercuric reductase	1205bp	66°C	Wang et al., 2011
merALR	5′-CGA GCY TKARSSCYT CGG MCAKSGTCA-3′				
czcAF	5′-TCG ACG GBGCC GTGGTSMTBGTC GAG AA-3′	Inner membrane protein contributing to Co, Zn, and Cd efflux (RND)	232bp	62°C	Roosa et al. (2014)
czcAR	5′-GTVAWSGCCAKCGGVBGGA ACA -3′				
czcCF	AGC CGY CAG TAT CCG GAT CTG AC	Outer part of a Co/Zn/Cd efflux protein (RND)	418bp	62°C	Roosa et al. (2014)
czcCR	GTG GTC GCC GCC TGA TAG GT				
czcDF1	5′-TCA TCG CCG GTG CGA TCA TCA -3′	single membrane polypeptide chemiosmotic efflux pump conferring resistance to Co, Zn, and Cd Cation diffusion facilitator	272bp	55°C	Roosa et al. (2014)
czcDR1	5′-TGT CAT TCA CGA CAT GAA CC-3′				
czcDF2	5′-TTT AGA TCT TTT ACC ACC ATG GGC GCA GGT CAC TCA CAC GAC C-3′	single membrane polypeptide chemiosmotic efflux pump conferring resistance to Co, Zn, and Cd	1000bp	60°C	Abou-Shanab et al. (2007)
czcDR2	5′-TTT CAG CTG AAC ATC ATA CCC TAG TTT CCT CTG CAG				

	CAA GCG ACTTC-3′				
nccAF1	5′-TTY AGC CAGGTVACSGTSATYTT-3′	Membrane protein conferring resistance to Co, Cd, and Ni	532bp	58°C	Roosa et al. (2014)
nccAR1	5′-GCY GCR TCSGCR CGC ACC AGR TA-3′				
***pbrT* (forward)**	AGC GCG CCC AGG AGC GCA GCG TCT T	Pb uptake protein	448bp	60°C	Roosa et al. (2014)
***pbrT* (reverse)**	GGC TCG AAG CCG TCG AGR TA				
nccAF2	ACGCCGGACATCACGAACAAG	Membrane protein conferring resistance to Co, Cd, and Ni	1141bp	62°C	Abou-Shanab et al. (2007)
nccAR2	CCAGCGCACCGAGACTCATCA				
mnxGIF	ACGCATGTCTTTCACTATCATGTTCAT	Mn(II) oxidation-associated multicopper oxidase gene	900bp	45°C	Francis et al. (2002)
mnxGIR	AAATAAGTGGTCATGGAAGAACCATGC				
smt1	GATCGACGTTGCAGAGACAG	Metallothionein gene	517bp	56°C	Naz *et al*. (2005)
smt2	GATCGAGGGCGTTTTGATAA				

4.2.8. Antibiotic susceptibility test

The antibiotic susceptibility test for the 20 selected isolates was carried out by the Kirby–Bauer method (Bauer et al., 1966) on Muller-Hinton agar plates (Hi Media, India). Selected isolates were enriched in Nutrient broth for 5 days and aseptically swabbed onto agar plates. The antibiotics tested were ampicillin (10 mcg), amoxycillin (30 mcg), nalidixic acid (30 mcg), tetracycline (30 mcg), neomycin (30 mcg), novobiocin (30 mcg), cefotaxime (30 mcg), penicillin-G (10 mcg), polymyxin-B (30 mcg), tobramycin (30 mcg), chloramphenicol (30 mcg), vancomycin (30 mcg), kanamycin (30 mcg), rifampicin (30 mcg), streptomycin (10 mcg), tetracycline (30 mcg), carbenicillin (100 mcg) and nitrofurantoin (300 mcg). The results were assessed after 5 days at 15°C incubation.

4.2.9. Statistical Analysis

Correlation between total heterotrophic bacterial counts and background metal concentrations was calculated to establish possible significant relationships. One-way ANOVA test and post hoc analysis (Tukey test) were used to compare the background metal concentrations and total heterotrophic bacterial count values between glacier, foreland meltwater channels, and fjord samples. Results were considered significant when $p < 0.05$. All data were square-root transformed for the analysis. Two-way ANOVA was performed to establish the influence of sample type (sediment/water) and sampling location (glacier/foreland/fjord) on the background metal concentrations. Additionally, the same Two-way ANOVA analysis was performed to verify the influence of sampling locations and metal concentrations on the bacterial counts obtained (PAST, version 3). Nonmetric multidimensional analysis (nMDS) analysis was performed to observe the clustering of the stations with respect to metal concentrations and bacterial counts from different amendments (Primer 6, Plymouth Marine Laboratory, United Kingdom).

4.3. Results

4.3.1. Trace metal analysis for the glacio-marine samples

The trace metal concentrations of the ice, water, and sediment samples are given in **Table 4.2**. The trace metal concentrations were higher for the sediment samples as compared to the ice and water samples. The highest concentration of Hg (90.9µg/Kg) and Pb (7900µg/Kg) were noted from the outer fjord sediment (KNS1 sed) while the highest concentration of Cd (300µg/Kg) and Ni (40800µg/Kg) were noted from the VB snout sediment. Glacier foreland sediment (BR sed) accounted for the highest concentration of Co (13200µg/Kg), Zn (223900µg/Kg), and Mn (362000µg/Kg) (**Table 4.2**). The lowest metal concentrations were observed in the VB ice and the Bayelva meltwater samples (**Table 4.2**). The trace metal concentrations obtained in the study were compared with the previously reported values from Svalbard (**Table 4.3 (a) and (b)**).

It was observed that the trace metal concentrations contributed to significant differences between the samples (ANOVA, $p < 0.05$). When we grouped the samples based on the sampling locations (VB (VB ice, and VB sediment), BR (BR water and BR sediment), and KNS (KNS1, KNS9, and KNBR water and sediments)) and checked their interaction with the different background metal concentrations using Two-way ANOVA, we could observe that at the 0.05 level, the interaction between sampling location and metal concentrations were not significantly different. While testing the interaction between the sample type (Terrestrial water- VB ice, BR 1, BR2, BR3, BR4, BR5; Terrestrial sediment- VB and BR sediment; Fjord water- KNS1, KNBR, and KNS9 water; Fjord sediment- KNS1, KNBR and KNS9 sediment) and background metal concentrations using Two-way ANOVA, we could observe significant interactions between sample type and metal concentrations ($p < 0.05$). It was also observed that at the 0.05 level, there was a significant difference between the water and sediment samples in terms of metal concentrations irrespective of the sampling locations. This was also evident from the nMDS plot (**Fig. 4.2**).

4.3.2. Estimation of total microbial counts and bacterial Viable Counts from different metal enrichments

The total microbial count for the glacier snout ice, snout sediments, foreland and fjord water and sediment samples are detailed in **Table 4.4**. The total heterotrophic bacterial counts on MM agar plates without enrichment were found to be in the order of 10^4 CFU/L for snout ice, 10^5 - 10^6 CFU/L for foreland MWs, and 10^6 CFU/L for fjord waters. Similarly, for the sediment samples, the total viable count was in the order of 10^5 CFU/g for the snout and foreland sediments while 10^6 CFU/g for the fjord sediments on MM agar plates without metal enrichment (**Table 4.4**).

The highest total retrievable heterotrophic bacterial count (RHBC) was noted for the Ni amendments (RHBC for water samples- 7.5×10^4 - 4×10^6 CFU/L; THBC for sediment samples-1.1×10^5 - 3.1×10^6 CFU/g) and Mn amendments (RHBC for water samples- 9×10^4 - 2.6×10^6 CFU/L; RHBC for sediment samples- 4.3×10^4 - 1.5×10^6 CFU/g) while the lowest count was noted for Hg amendments (RHBC for water samples- 2×10^3 - 6.5×10^4 CFU/L; RHBC for sediment samples - 10^3 CFU/g). No bacterial growth was noted from samples enriched with a combination of Hg, Pb, and Cd while for the samples enriched with a combination of Ni, Co, Zn and Mn, the RHBC values varied between 1.5×10^3 - 2×10^5 CFU/L for water samples and 5.5×10^4- 6×10^4 CFU/g for sediment samples. Similarly, for the VB ice sample, colonies were grown only in nickel enrichment (1.5×10^5 CFU/L). It was also noted that the fjord water and fjord sediments had higher heterotrophic bacterial counts for different metal enrichments as compared to the snout ice, snout sediment, and foreland water and sediments (**Table 4.4**).

Table. 4.2.
Trace metal concentrations from all the sampling locations.

Station Details	Hg (µg/Kg)	Pb (µg/Kg)	Cd (µg/Kg)	Ni (µg/Kg)	Co (µg/Kg)	Zn (µg/Kg)	Mn (µg/Kg)
VB ice	0	0.008	0.029	0.37	0.02	7.77	0.59
BR1 water	0	0.015	0.037	0.03	0.01	1.04	0.08
BR2 water	0	0.026	0.053	1.85	0.01	18.81	0.94
BR3 water	0	0.0097	0.024	0.19	0.04	9.98	0.36
BR4 water	0	0.010	0.031	0.16	0.03	4.46	2.32
BR5 water	0	0.011	0.032	0.36	0.02	2.6	1.15
KNS1 water	3.6	0.0015	0.052	6.8	0.68	355.22	8.71
KNBR water	3.05	0.037	1.16	9.99	0.79	321.89	10.14
KNS9 water	3.3	0.0024	0.001	5.55	0.32	483.72	8.41
VB snout sediment	44.8	3900	300	40800	8300	166700	205300
BR sediment	18.2	2900	100	16100	13200	223900	362000
KNS1 sediment	90.9	7900	200	21300	11700	136100	314100
KNBR sediment	38.6	3000	200	20800	9400	152900	250500
KNS9 sediment	22.1	2600	100	26600	11900	117300	332600

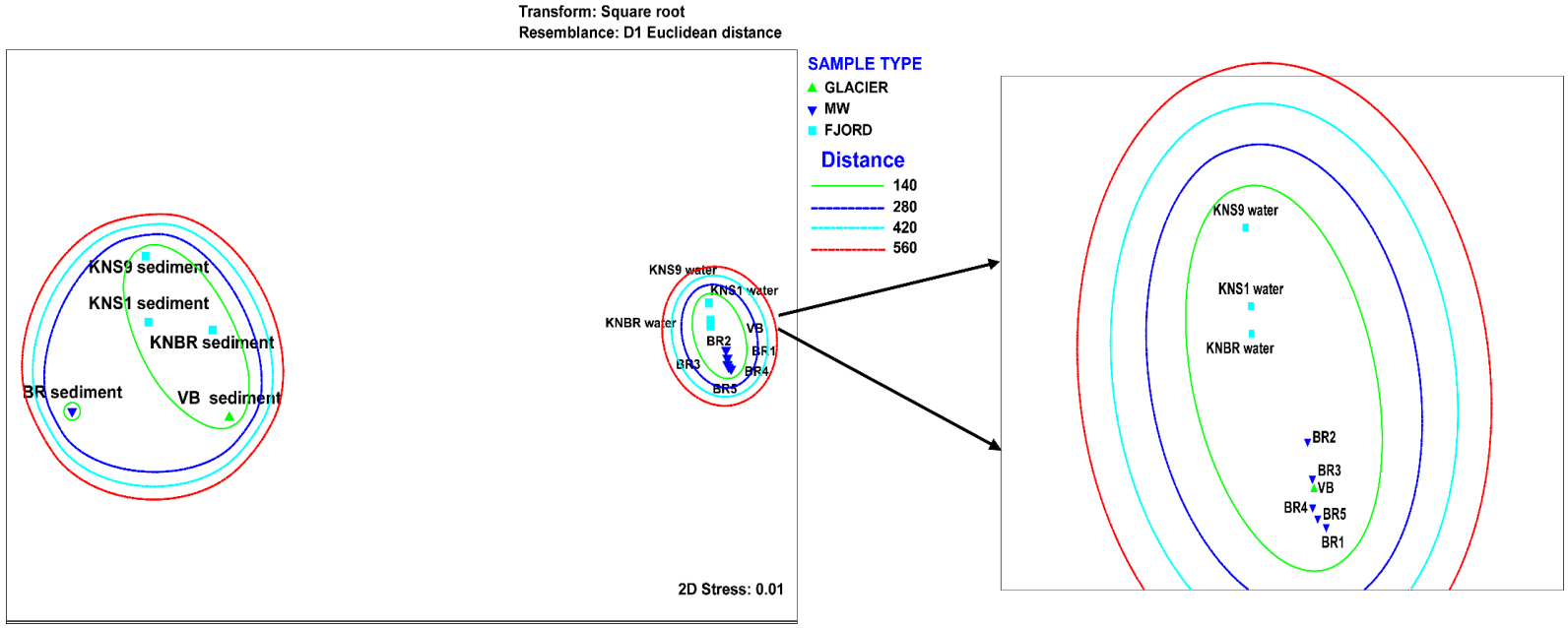


Fig. 4.2. nMDS plot showing the sample distribution based on the background trace element concentrations

Table 4.3a.

Comparison of trace metal values from Vestrebroggerbreen glacier associated and Kongsfjorden sediments with previous reports.

Location	Ni	Co	Mn	Zn	Cd	Pb	Hg
Glacier-fjord sediments (**Present study**) **(µg/g)**	Glacier: 16.1-40.8 Fjord: 20.8-26.6	Glacier: 8.3-13.2 Fjord: 9.4-11.9	Glacier:205.3-362 Fjord: 250.5-332.6	Glacier: 166.7-223.9 Fjord: 117.3-152.9	Glacier: 0.1-0.3 Fjord: 0.1-0.2	Glacier: 2.9-3.9 Fjord:2.6-7.9	Glacier: 0.018-0.04 Fjord: 0.02-0.09
Surface sediments from Kongsfjorden **(Lu et al., 2012) (µg/g)**	29.98 **BV**: 26.22	15.08 **BV**: 13.56	440.03 **BV**: 364.08	85.50 **BV**: 70.49	0.19 **BV**: 0.14	22.60 **BV**: 17.46	0.022 **BV**: 0.0098
Surface sediments from Kongsfjorden (**Singh et al., 2015**) **(µg/g)**	30.18	11.88	322. 57	100.82	0.18	16.42	-
Surface sediments from Kongsfjorden (**Grotti et al., 2013) (µg/g)**	41	12	446	75	0.31	18	-
Surface sediments from Kongsfjorden (**Grotti et al., 2017**) **(µg/g)**	31	11	485	75	0.3	14	-
Surface sediments from Kongsfjorden (**Mohan et al., 2018**) **(µg/g)**	-	-	-	-	-	-	0.48
Surface soils from Ny-Alesund (**Hao et., 2013**) **(µg/g)**	15.47 **BV**: 6.24	-	-	78.85 **BV**: 77.3	0.02 **BV**: -	9 **BV**: 4.38	0.28 **BV**: 0.27
Surface soils from Bayelva area, Ny-Alesund (**Halbach et al., 2017**) **(µg/g)**	12.9±5.9	-	250±123	73.2±14.9	0.571±0.18	14.3±4.5	0.122±0.043
Surface sediments from Kongsfjorden (**Choudhary et al., 2020**)	29.47	17.9	600	77.2	0.16	14.11	-

BV- baseline value

Table 4.3b.

Comparison of trace metal values of Vestrebroggerbreen glacier associated and Kongsfjorden waters with previous reports.

Location	Ni	Co	Mn	Zn	Cd	Pb	Hg
Glacier-fjord waters (**Present study**) **(µg/Kg)**	Glacier: 0.03-1.85 Fjord: 5.5-9.9	Glacier: 0.01-0.04 Fjord: 0.32-0.79	Glacier: 0.08-2.32 Fjord: 8.41-10.14	Glacier:1.04-18.81 Fjord: 321.9-483.7	Glacier: 0.023-0.052 Fjord: 0.001-1.16	Glacier: 0.008-0.02 Fjord: 0.001-0.03	Glacier: 0 Fjord: 3.04-3.6
Bayelva waters (**Moe. 2018**) **(µg/Kg)**	0.101-2.05	-	2.04-12.2	0.0489-136	0.00401-0.0245	0.00577-1.33	0-0.00770
Adventfjorden waters (**Kalinowska et al., 2020**) **(µg/Kg)**	0.06-1.01	0.02-0.32	0.14-3.88	0.56-3.35	0.01-0.04	0.09-0.13	-
Typical values found in marine waters (**Moore.1981; Muir et al., 1992; Glabonjat et al., 2018**) **(µg/Kg)**	0.1-0.3	0.003-7.7	0.01-1.1	0.07-2.14	0.01-0.07	0.01-0.05	-

Table. 4.4.
Total microbial counts and viable counts on control as well as metal-enriched samples from different sampling locations.

Sampling Location	**Station Details**	**Total Counts (cells/L)**	**Viable count on NA Plates without metal after enrichment (CFU/L)**	**Viable count on metal amended plates (CFU/L)**								
				Hg	**Pb**	**Cd**	**Ni**	**Co**	**Zn**	**Mn**	**C1 (Hg+Pb+Cd)**	**C2 (Ni+Co+Zn+Mn)**
Glacier snout	**VB ice**	5.4×10^{7}	5×10^{4}	-	-	-	1.5×10^{5}	-	-	-	-	-
Glacier foreland	**BR1 water**	6.2×10^{7}	2×10^{5}	5×10^{3}	9×10^{3}	3.6×10^{4}	9.4×10^{4}	-	-	1.1×10^{5}	-	-
	BR2 water	7.4×10^{7}	1.1×10^{5}	2×10^{3}	1.5×10^{5}	1.1×10^{5}	1.3×10^{5}	-	-	1.3×10^{5}	-	-
	BR3 water	6.7×10^{7}	8×10^{5}	-	-	-	-	6.3×10^{4}	5×10^{3}	1.1×10^{5}	-	-
	BR4 water	6.3×10^{7}	5×10^{5}	-	1×10^{4}	1.1×10^{5}	1.2×10^{5}	1.5×10^{5}	2×10^{4}	-	-	1.2×10^{5}
	BR5 water	6.5×10^{7}	1.5×10^{6}	3.8×10^{4}	7×10^{4}	4.5×10^{4}	1.1×10^{5}	2.5×10^{4}	3×10^{4}	9×10^{4}	-	1.5×10^{3}
Fjord (Outer)	**KNS1 water**	7.2×10^{8}	5×10^{6}	5.6×10^{4}	1.1×10^{5}	1.2×10^{5}	4×10^{6}	1.5×10^{5}	1×10^{5}	2.6×10^{6}	-	7.5×10^{3}
(Middle)	**KNBR water**	5.5×10^{8}	4.3×10^{6}	6.5×10^{4}	1.1×10^{5}	1.2×10^{5}	1.5×10^{6}	8×10^{3}	1.4×10^{5}	1.75×10^{6}	-	3.5×10^{4}
(Inner)	**KNS9 water**	6.1×10^{8}	3×10^{6}	-	3.4×10^{4}	5×10^{4}	7.5×10^{4}	5.5×10^{4}	1×10^{5}	1.45×10^{5}	-	2×10^{5}
Sampling Location	**Station Details**	**Total Counts (cells/g)**	**Viable count on Nutrient Agar Plates (CFU/g)**	**Viable count on metal amended plates (CFU/g)**								
				Hg	**Pb**	**Cd**	**Ni**	**Co**	**Zn**	**Mn**	**C1 (Hg+Pb+Cd)**	**C2 (Ni+Co+Zn+Mn)**
Glacier snout	**VB snout sediment**	6.2×10^{6}	3.4×10^{5}	-	1.7×10^{5}	-	1.1×10^{5}	1×10^{3}	-	-	-	-
Glacier foreland	**BR sediment**	4.3×10^{6}	5×10^{5}	9×10^{3}	2×10^{5}	1.1×10^{5}	1.6×10^{5}	7.4×10^{4}	-	1.5×10^{5}	-	-
Fjord (Outer)	**KNS1 sediment**	4.9×10^{8}	5.6×10^{6}	-	5.3×10^{5}	7.5×10^{5}	3.1×10^{6}	-	1.4×10^{4}	1.5×10^{6}	-	-
(Middle)	**KNBR sediment**	2.2×10^{8}	3×10^{6}	-	8.6×10^{5}	1×10^{4}	1.5×10^{6}	1.3×10^{5}	2×10^{3}	1.8×10^{5}	-	5.5×10^{4}
(Inner)	**KNS9 sediment**	4.7×10^{8}	4.7×10^{6}	-	2.9×10^{5}	4.1×10^{4}	1×10^{6}	1.3×10^{5}	1.4×10^{4}	4.3×10^{4}	-	6×10^{4}

4.3.3. Viable Counts versus Metal Concentrations, sampling locations, and sample types

One-way ANOVA analysis indicated that the viable count values for different metal amendments and different sampling locations are significantly different ($p<0.05$). Two-way ANOVA analysis indicated that the influence of sampling location, sample type, and type of metals on viable count was significantly different ($p<0.05$). Similarly, the interaction between sampling location and metals as well as between sample type and metals on viable count was significant ($p<0.05$). Significant differences in the viable counts between KNS and VB samples as well as between KNS and BR ($p<0.05$) were observed, while there was no significant difference between VB and BR samples. Similarly, there were significant differences between the viable counts of terrestrial water-fjord water, terrestrial sediment-fjord sediment, terrestrial water-fjord sediment, terrestrial sediment-fjord sediment ($p<0.05$), while there was no significant difference noted between the viable counts of terrestrial water- terrestrial sediments and fjord water-fjord sediments as per the two-way ANOVA. This was supported by the nMDS results (**Fig. 4.3**). From the Pearson correlation analysis, we could also note significant correlation between the viable count on Pb amendment to that of the background Pb concentration ($p=0.02178$).

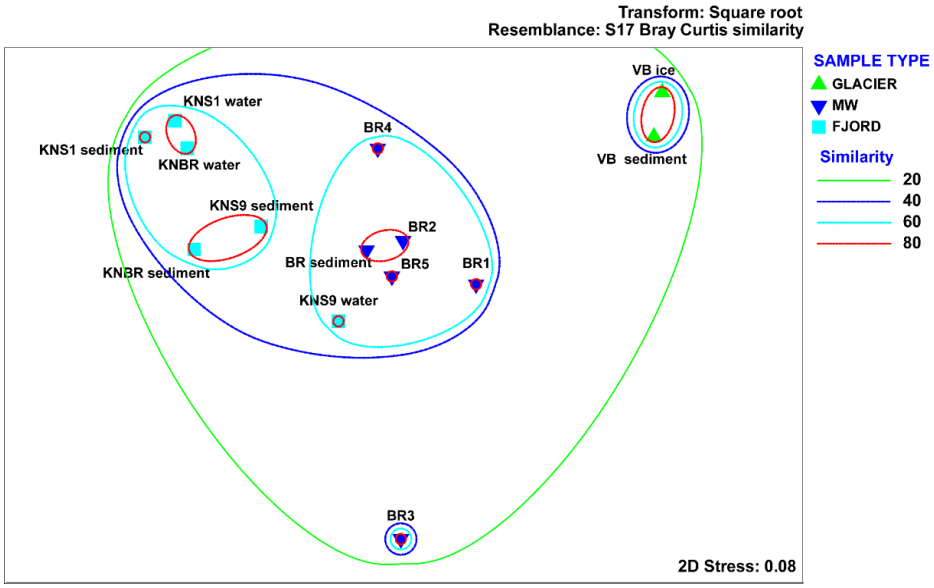


Fig. 4.3. nMDS showing the sample distribution based on the total heterotrophic bacterial count from different metal amendments

4.3.4. Identification and characterization of metal tolerant bacterial strains

317 morphologically distinct bacterial colonies were selected from the metal enrichment experiment and sub-cultured onto different metal amended plates. This resulted in the selection of 202 isolates that grew well on the metal amended plates. Rep-PCR analysis helped to further shortlist 106 bacterial isolates for 16SrRNA gene sequencing. The 106 bacterial isolates identified from the metal-enriched media belonged to 55 different bacterial species and 27 different genera representing the phylum Proteobacteria (Class α-, β- and γ-proteobacteria), Actinobacteria, Bacteroidetes, and Firmicutes. Among the 106 bacterial sequences, a total of 98 identified 16S rRNA gene sequences (sequence length >1200bp) belonging to the isolated unique strains were deposited in GenBank under the accession numbers **MZ311755-MZ311852**.

Class γ-proteobacteria was dominant among all samples irrespective of the sampling locations, type of samples, and type of metals. While looking at the bacterial diversity based on sampling locations, we could observe the presence of Class γ-, α-, and β-proteobacteria in all the samples. γ-proteobacteria was dominant in the snout and fjord samples while Actinobacteria was dominant in the Bayelva samples. Class Flavobacteriia was noted only in the fjord samples (**Fig. 4.4**). Similarly, while considering the diversity based on the type of samples (water/sediment), we could observe that species belonging to Class Bacilli and Class Flavobacteriia were isolated only from the sediment samples (**Fig. 4.4**). Similarly, from the bacterial isolation results based on the type of metals, we could observe the dominance of γ-proteobacteria in all the 7 metal amendments and their combinations. The abundance of Actinobacteria and α-proteobacteria was noted from 7 out of 8 metal amendments (**Fig. 4.4**). The presence of Flavobacteriia was noted only from the Pb amended plates.

The highest number of metal tolerant bacterial species were retrieved from the fjord sediments (24 species) while the lowest number of bacterial species were noted from the glacier snout ice sample (3 species). Different species belonging to the genus *Pseudomonas* were noted from all samples irrespective of the sampling location (**Table 4.5**).

Polaromonas and *Janthinobacterium* species were found to be specific to both VB and BR samples while *Pseudarthrobacter* species were specific to only BR water and BR sediment samples. Similarly, *Salinibacterium, Halomonas, Pseudoalteromonas,* were fjord-specific species isolated from both fjord water and sediment samples while *Psychrobacter, Planococcus, Hoeflea,* and *Sulfitobacter* species were retrieved only from fjord sediments. Species closely related to *Brucella intermedia* (earlier classified as *Ochrobactrum intermedium)* were isolated from VB, BR, and KNS water samples. However, there were no species isolated from all three (VB, BR, and KNS) sediment samples (**Table 4.5**).

While taking into consideration the different metal enrichments, the highest number of bacterial species were noted from the Mn and Ni amendments (16 and 13 species) while the lowest number of species was noted for Hg amendments (4 species). *Pseudomonas* species were retrieved from all different metal enrichments and also from the trace metal combination while the presence of *Halomonas* and *Psychrobacter* species were noted from 7 out of 8 metal amendments. The bacterial genus *Staphylococcu*s was retrieved from Hg, Cd, and Mn enrichments. Similarly, there were bacterial genera specific to each metal amendment which included *Dietzia* sp. from Hg, *Arenibacter* sp. from Pb, *Sphingomonas*, *Methylobacterium*, and *Sulfitobacter* sp. from Cd, *Microbacterium* sp. from Zn, *Pararhizobium* and *Micrococcus* sp. from Ni, *Marinomonas* sp. from Co, and *Paraglaciecola* sp. from trace metal combination (**Table 4.5**).

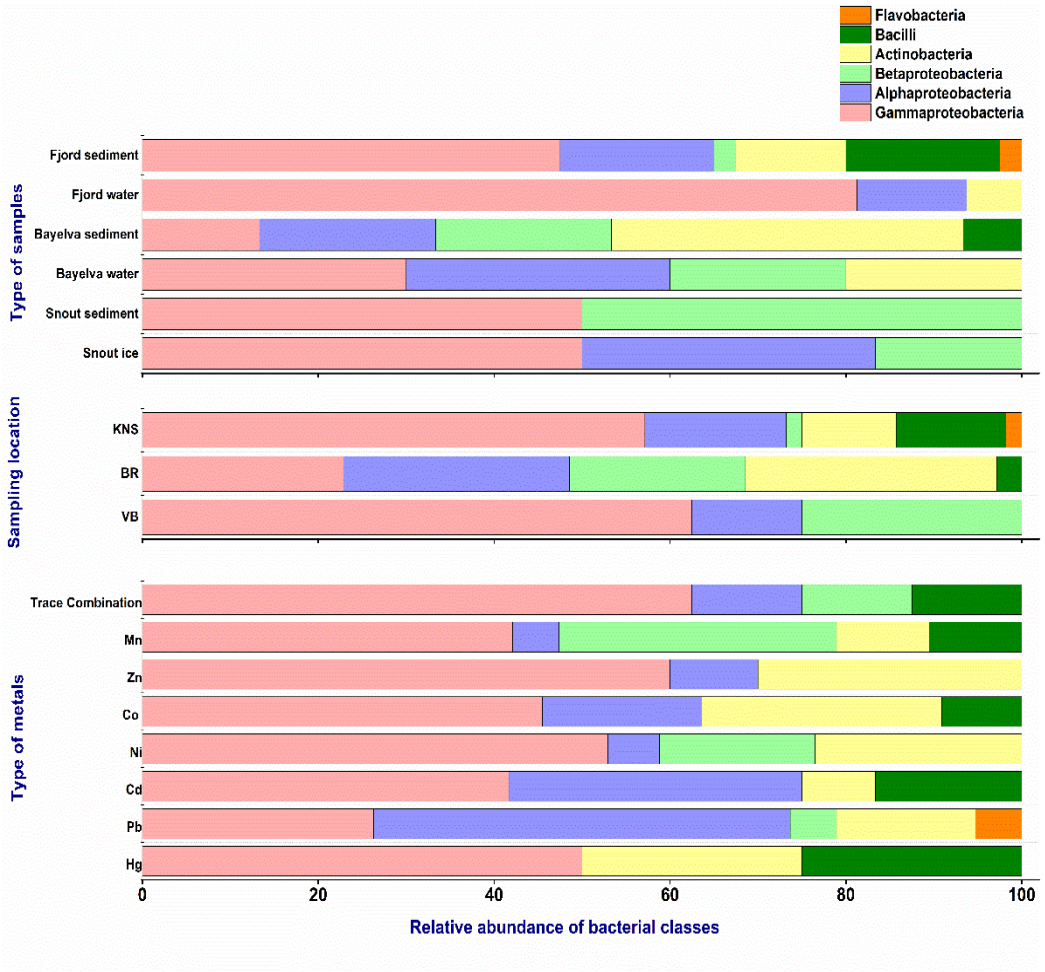


Fig. 4.4. Relative abundance of bacterial classes grouped based on sample type, sampling location, and metal amendment.

Table 4.5.

List of retrievable heterotrophic bacterial isolates from the glacier snout, glacier foreland, and Kongsfjorden water and sediments obtained from different metal amendments along with their taxonomic affiliation and closest type strain-description.

Strain Name	Source of isolation	Metal amendment	Closest relative type strain	Sequence length	% similarity	Isolation source/ Description
			VB (Glacier snout)			
HM78	VB Ice	Ni	*Polaromonas glacialis* (HM583568)	1403bp	99.29	Alpine glacier cryoconite, Arctic glacier foreland
HM187, HM184, **HM193**	VB Ice	Hg	*Pseudomonas fildesensis* (MK859934)	1397bp	99.93	From soil sample, King George Island, Antarctica
HM186, HM185	VB Ice	Pb	*Brucella intermedia* (ACQA01000003)	1357bp	100	From human blood
HM157, **HM137**	VB sed	Ni, Mn	*Pseudomonas silesiensis* (KX276592)	1413bp	99.57	From pesticide exploited peaty soil in a biological wastewater treatment plant
HM136	VB sed	Mn	*Janthinobacterium lividum* (Y08846)	1400bp	99.71	red/purple pigment production-prodigiosin/violacein. The Antarctic strain found to have genes for antimicrobial compounds, antibiotic resistance, and metal resistance
HM156	VB sed	Mn	*Polaromonas cryoconiti* (HM583567)	1409bp	99.29	Alpine glacier cryoconite
			BR (Glacier foreland)			
HM66	BR1	Hg	*Dietzia timorensis* (LMTB01000088)	1345bp	99.85	soil, Timor Indonesia, Genus known for biodegradation of organic carbon
HM67	BR1	Pb	*Pseuarthrobacter oxydans* (MN559964)	1395bp	98.78	Antarctic soil collected from the Cape Burk area reported for Hg tolerance-Arctic tundra

HM68	BR1	Cd	*Sphingomonas paucimobilis* (BBJS01000072)	1320bp	99.85	Human clinical specimens, Pathogenic, High Cd tolerance (up to 200mg/L), Cu resistant genes
HM69, HM70	BR1	Cd	*Methylobacterium oryzae* (CP003811)	1349bp	100	Stem tissues of rice, Facultatively methylotrophic, Reducing toxicity of Ni & Cd in tomato
HM80	BR1	Ni	*Micrococcus luteus* (CP001628)	1317bp	99.7	resuscitation-promoting factor (Rpf) produced- boost degradation of contaminants in wastewater
HM150	BR sed	Ni	*Pararhizobium herbae* (GU565534)	1351bp	99.7	From root nodules of herb legume, China
HM148	BR sed	Ni	*Pseudarthrobacter sulfonivorans* (AF235091)	1377bp	99.41	Facultative methylotroph, dimethylsulfone metabolism, degrade crude oil and multi benzene compounds
HM147	BR sed	Mn	*Polaromonas glacialis* (HM583568)	1402bp	99	Alpine glacier cryoconite, Arctic glacier foreland,
HM180	BR2	Zn	*Microbacterium xylanilyticum* (AJ853908)	1401bp	97.92	From biofilm, degrades Xylan
HM177, **HM178**, HM65	BR2, BR5	Co, Pb	*Brucella intermedia* (ACQA01000003)	1357bp	99.93	From human blood
HM129	BR sed	Pb	*Arthrobacter oryzae* (AB279889)	1394bp	99.64	From paddy soil sample, Japan
HM130, HM131**,** HM139, HM142, **HM146**	BR sed, BR5	Cd, Ni, Hg, Co	*Pseudomonas silesiensis* (KX276592)	1411bp	99.93	From pesticide exploited peaty soil in a biological wastewater treatment plant
HM132, HM133	BR sed	Mn	*Pseudarthrobacter sulfonivorans* (AF235091)	1390bp	99.2	facultative methylotroph, dimethylsulfone metabolism, can degrade crude oil and multi benzene compounds
HM50	BR sed	Hg	*Staphylococcus arlettae* (AB009933)	1371bp	99.93	From skin of horses, Novobiocin-resistent, Penicillin-resistent, novel beta lactamase present.

HM47	BR sed	Pb	*Arthrobacter citreus* (X80737)	1386bp	99.28	Ability to degrade phenol
HM45	BR sed	Ni	*Arthrobacter glacialis* (JX949500)	1389bp	99.5	Glaciers in China
HM52, **HM114**	BR sed	Mn	*Polaromonas glacialis* (HM583568)	1399bp	98.71	Alpine glacier cryoconite, Arctic glacier foreland,
HM102, HM100, HM212, **HM209**	BR4	Pb, Ni, Mn	*Janthinobacterium svalbardensis* (DQ355146)	1399bp	99.79	Svalbard glacier ice, Violacein pigment-producing bacteria - defense against radiation, antimicrobial and anticancerous properties
HM63	BR5	Comb	*Pseudomonas mandelii* (BDAF01000092)	1385bp	99.86	Natural mineral waters, genes for the reduction of nitrate to dinitrogen
HM 140, HM141	BR5	Cd, Ni	*Pseudomonas fildesensis* (MK859934)	1403bp	99.93	From soil samples from King George Island, Antarctica
HM111, HM110	BR sed	Pb	*Devosia limi* (LAJF01000157)	1346bp	98.51	isolated from a nitrifying inoculum
KNS (Kongsfjorden)						
HM88, **HM2**	KNBR sed	Pb, Zn	*Paracoccus sediminilitoris* (MH491014)	1334bp	97.22	From tidal flat sediment, East China Sea
HM87, **HM99**	KNBR sed, KNS1 sed	Cd, Mn	*Staphylococcus warneri* (L37603)	1429bp	99.93	pathogenic
HM1, **HM115**	KNBR sed, KNS9 sed	Zn, Comb	*Psychrobacter nivimaris* (AJ313425)	976bp	99.9	The Southern Ocean. Attached to organic particles
HM86	KNBR sed	Ni	*Psychrobacter piscatorii* (AB453700)	1377bp	99.56	From fish processing plant
HM84, HM13	KNBR sed, KNS1 sed	Co, Zn	*Psychrobacter fozii* (AJ43082	1399bp	94.77	Antarctic coastal marine environments
HM3, HM5	KNBR sed	Mn	*Pseudoalteromonas arctica* (CP011026)	1392bp	99.78	Seawater sample, Spitzbergen, Arctic
HM32, HM123, HM154, HM15, HM16	KNBR , KNS1 sed, KNS1, KNS9	Ni, Pb, Co	*Pseudoalteromonas translucida* (CP011034)	1405bp	99.57	Seawater, sea of Japan. Fe transport system, multidrug resistance

HM33	KNBR	Co	*Marinomonas primoryensis* (AB074193)	1412bp	99.43	Marine coastal sea-ice in the sea of Japan, Ca^{2+} dependant hyperactive Anti-freeze protein (AFP) reported
HM75, HM76, HM171, HM166, HM118, **HM170**	KNS1 sed, KNS1	Pb , Zn, Mn, Cd	*Halomonas titanicae* (AOPO01000038)	1401bp	100	From rusticles from RMS Titanic. Metal corrosion genes present, Fe uptake regulators, Fe binding proteins
HM11	KNS1 sed	Pb	*Psychrobacter glaciei* (FJ748508)	1402bp	99.86	ice core of Austre Lovénbreen in Ny-Ålesund, Svalbard
HM77, **HM116**, HM60, HM98	KNS1 sed, KNS9 sed, KNS9	Cd, Mn, Comb	*Halomonas neptunia* (AF212202)	1399bp	99.57	Deep-sea hydrothermal vent environment, Pacific Ocean
HM128	KNS1 sed	Zn	*Pseudomonas pelagia* (AROI01000066)	1400bp	98.85	culture of the Antarctic green alga
HM19	KNS1 sed	Zn	*Psychrobacter cryohalolentis* (CP000323)	1405bp	99.43	Siberian Permafrost
HM23	KNS1 sed	Zn	*Psychrobacter okhotskensis* (AB094794)	1402bp	100	Monbetsu coast of the Okhotsk Sea in Japan, when ice carried by the cold current came to the area
HM152	KNS1 sed	Ni	*Pseudoalteromonas distincta* (JWIG01000030)	1403bp	99.93	Marine sponge, seawater, Amursky Bay, Pacific ocean
HM71	KNS1 sed	Ni	*Hoeflea alexandrii* (AJ786600)	1300bp	98.62	From toxic dinoflagellate
HM95, HM96, HM168, **HM38**, HM26, HM197	KNS1 sed, KNS1, KNS9 sed	Co, Ni, Zn, Cd	*Salinibacterium amurskyense* (AF539697)	1389bp	99.86	Seawater, Gulf of Peter, East sea
HM169, **HM12**, HM27	KNS1 sed, KNS9 sed	Co, Mn, Cd	*Planococcus halocryophilus* (ANBV01000012)	1422bp	100	Permafrost active layer soil, Canadian high Arctic, extreme sub-zero sp.
HM53, **HM9**	KNS1 sed	Mn, comb	*Planococcus halotolerans* (MH266202)	1416bp	98.44	Saline soil sample in China, produces rhamnolipid having antimicrobial activity
HM92	KNS1 sed	Mn	*Devosia euplotis* (AJ548825)	1358bp	98.28	Bacterial endosymbiont inhabiting cytoplasm of marine ciliated protozoon

HM8	KNS1 sed	Comb	*Polaromonas naphthalenivorans* (CP000529)	1031bp	99.22	Coal tar contaminated freshwater, naphthalene degrading bacteria
HM126	KNS1	Mn	*Pseudoalteromonas paragorgicola* (AY040229)	1327bp	99.53	From sponges
HM124	KNS1	Comb	*Pseudoalteromonas elyakovii* (AF082562)	1405bp	99.93	From marine molluscs
HM93	KNS1	Comb	*Devosia naphthalenivorens* (MG230310)	1218bp	98.43	Sediment sample, East Pacific Ocean,
HM119	KNS1	Comb	*Paraglaciecola polaris* (BAER01000081)	1238bp	98.35	Arctic ocean
HM24	KNS9 sed	Pb	*Arenibacter catalasegens* (MG183691)	1388bp	99.27	Surface sediment, Southern Indian ocean
HM194, HM195	KNS9 sed	Pb	*Hoeflea marina* (AY598817)	1343bp	99.7	Seawater, Germany
HM18	KNS9 sed	Cd	*Sulfitobacter litoralis* (jgi.1058904)	1333bp	98.17	Marine bacteria from the East sea, Korea
HM21	KNS9 sed	Mn	*Pseudoalteromonas prydzensis* (U85855)	1407bp	99.43	Antarctic sea ice,
HM61	KNS9	Pb	*Brucella intermedia* (ACQA01000003)	1351bp	100	From human blood

Comb-combination of metals

4.3.5. Distribution of the heavy metal-resistance genes in the analyzed pools of bacteria

The bacterial DNA isolated from each of the 98 isolates was used as the templates in PCR-based detection of metal-resistance genes in bacterial genomes which included *merA*, *czcA*, *czcC*, *czcD*, *smt*, *mnx*, and *nccA* (as detailed in **Table 4.1**). The specific PCR products were obtained for four genes, i.e., *merA*, *czcA*, *czcC*, and *czcD* (**Fig.4.5**). In total, 47 metal-resistance genes were identified. The highest number of gene sequences (25) was noted for the *czcC* gene while 5, 6, and 11 gene sequences were noted for *czcD, czcA,* and *merA* genes respectively. 16 gene sequences belonged to isolates retrieved from Kongsfjorden water and sediments (8 from outer fjord, 4 each from middle and inner fjord) while 29 sequences belonged to isolates retrieved from glacier foreland samples (24 from meltwaters, 5 from sediments). Only 2 identified metal gene sequences belonged to isolates retrieved from the glacier snout samples. Isolate HM 157 closely related to *Pseudomonas silesiensis* (**Fig. 4.6**) was shown to contain three out of four identified genes.

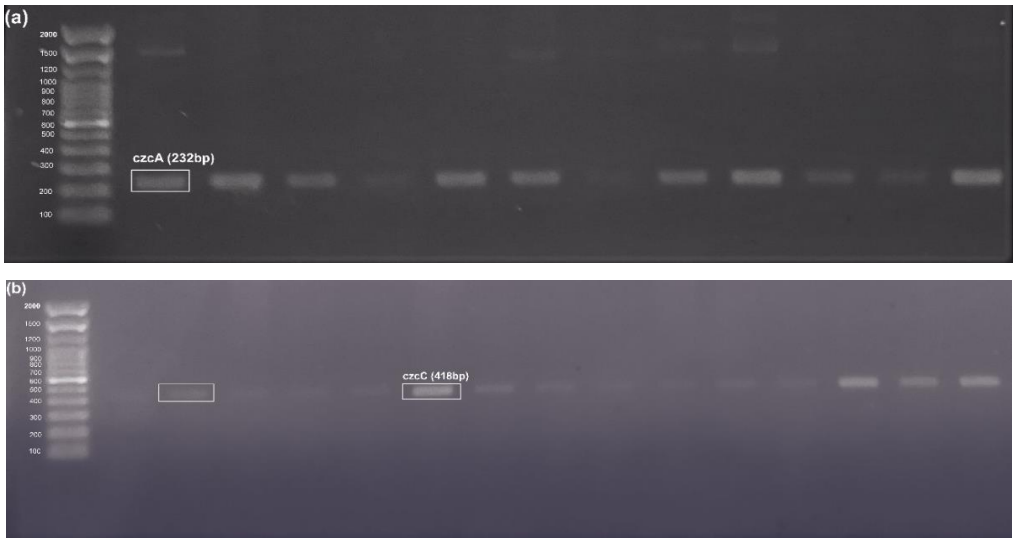
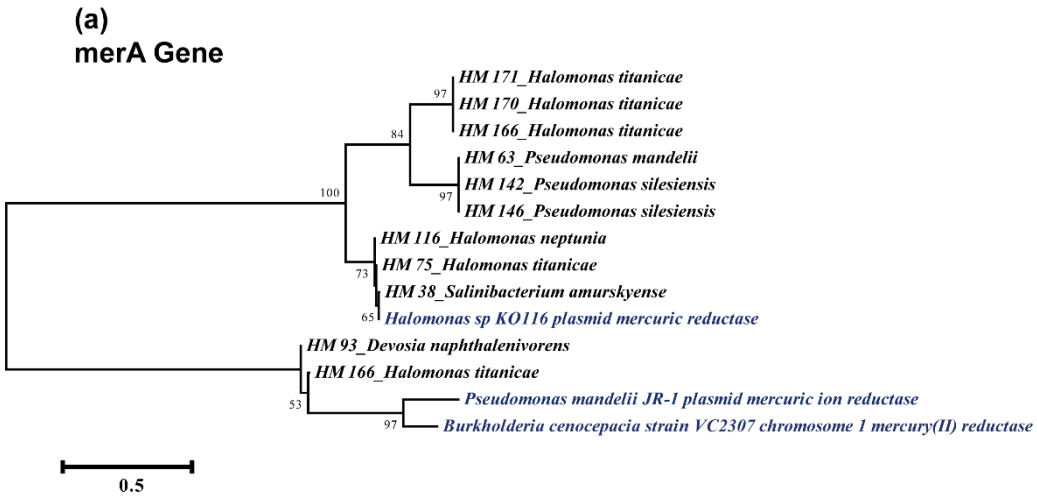
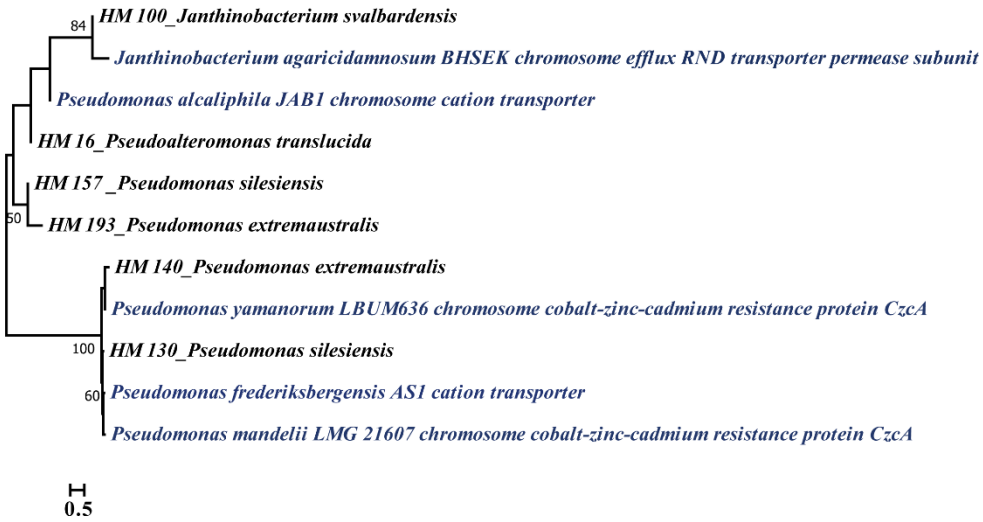


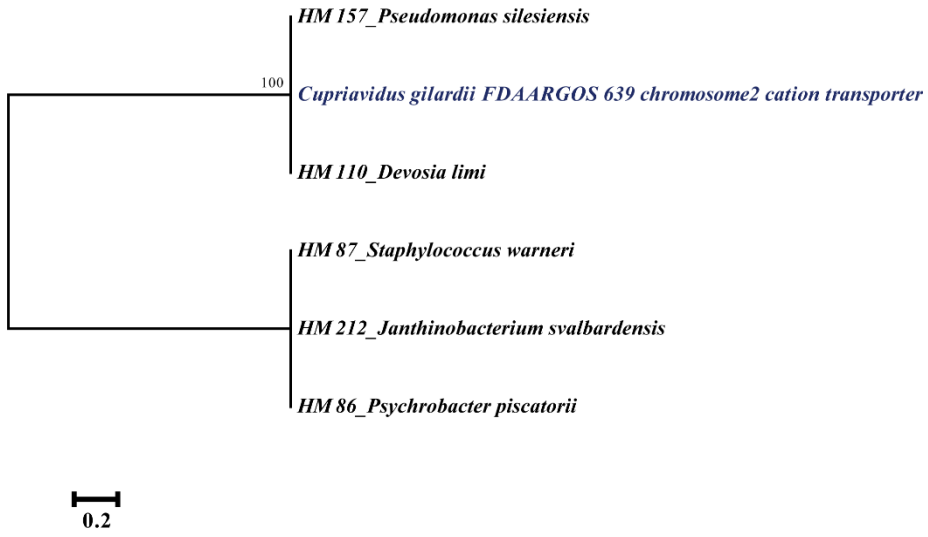
Fig. 4.5. Agarose gel electrophoresis image of **(a)** *czcA* gene PCR amplified product (*czcA* amplicon size-232bp), **(b)** *czcC* gene PCR amplified product (*czcC* amplicon size-418bp), **(c)** *czcD* gene PCR amplified product (*czcD* amplicon size- 1000bp), **(d)** *merAL* gene PCR amplified product (*merAL* amplicon size - 1205bp). Lane 1 is the Marker lane.



(b)
czcA Gene



(c)
czcD Gene



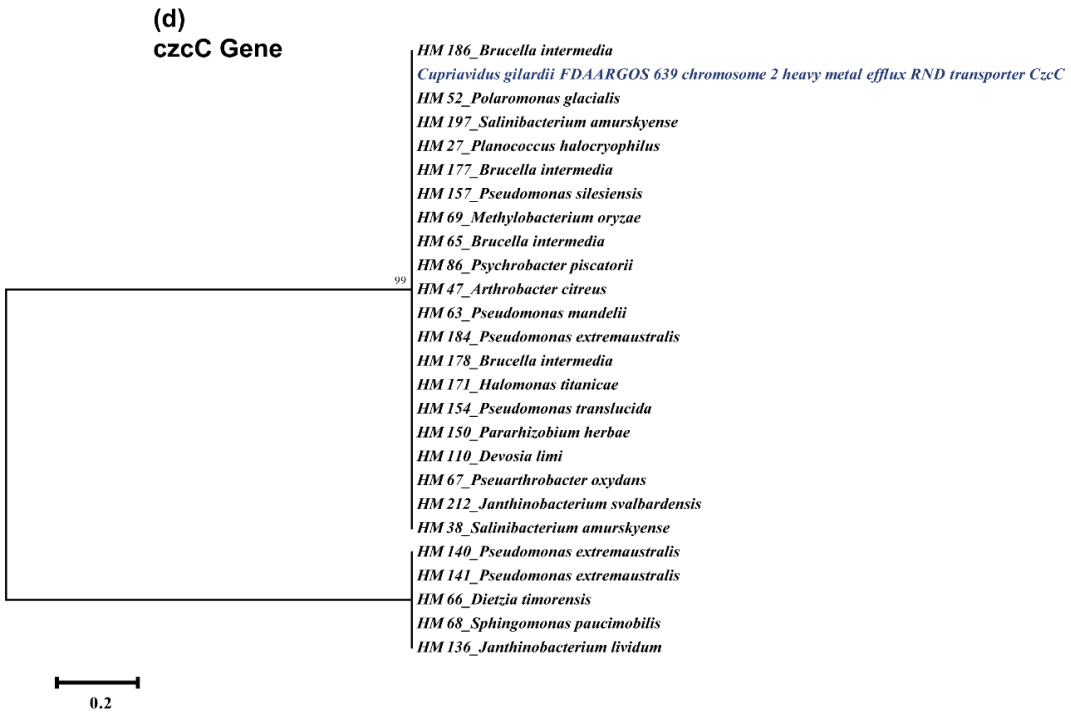


Fig. 4.6. The phylogenetic trees showing the diversities of **(a)** *merA*, **(b)** *czcA*, **(c)** *czcD***,** and **(d)** *czcC* genes detected in different metal tolerant Arctic bacteria.

4.3.6. Secondary screening of metal tolerant bacterial fraction and determination of MIC

When subjected to secondary screening to higher metal concentrations in broth media (Hg (5-10µM), Cd (50-100µM), Pb (50-100µM), Ni (200-500µM), Co (200-500µM), Zn (250-500µM), and Mn (2.5-10mM)) and screening for multi-metal tolerance to all 7 metals and metal combinations, 20 bacterial isolates were short-listed out of the total 106, which were multi-metal tolerant. The 20 isolates belonged to *Psychrobacter, Halomonas, Planococcus, Pseudomonas, Brucella, Hoeflea, Devosia, Salinibacterium, Staphylococcus, Rhodococcus,* and *Dietzia* species (**Fig. 4.7**).

Determination of the maximum tolerable metal concentration for each metal for the 20 isolates and their antibiotic resistance properties were evaluated. From the results, it was observed that Hg is more toxic as compared to the other metals since the highest concentration that the bacteria could tolerate was 10µM. The highest concentration of Cd and Pb tolerated by the isolates was 250 µM while the highest concentration of Ni, Co, and Zn tolerated by the isolates was up to 1mM. Similarly up to 10mM of Mn was

tolerated by the bacterial isolates (**Fig. 4.7**). Among the 20 isolates, HM11 (closely related to *Psychrobacter glaciei*, 99.86%) exhibited the highest tolerance level towards the different metals (Hg - 10µM, Pb and Cd - 250µM, Ni, Co, and Zn - 1mM and Mn - 10mM). HM116 (closely related to *Halomonas neptunia* (99.57%) exhibited metal tolerance in the range: Hg - 5µM, Pb and Cd - 150µM, Ni, Co and Zn - 1mM and Mn - 10mM) while HM12 (closely related to *Planococcus halocryophilus* (100%) exhibited metal tolerance in the range: Hg - 5µM, Pb - 100µM and Cd - 150µM, Ni, Co, and Zn - 500µM, and Mn -10mM) (**Fig. 4.7**).

4.3.7. Antibiotic resistance properties of metal tolerant bacteria

It was observed that 18 out of 20 bacterial isolates tested showed resistance to at least 2 antibiotics tested while only one isolate (HM100, closely related to *Janthinobacterium svalbardensis*, 99.86%) was susceptible to all of the antibiotics tested. Bacterial isolate HM92 (*Devosia euplotis*, 98.28%) exhibited tolerance to 12 antibiotics tested while the isolate HM128 (*Pseudomonas pelagia*, 98.85%) exhibited tolerance to 10 different antibiotics tested. Isolates HM11, HM12, and HM116 exhibited resistance towards 1, 2, and 3 antibiotics that were tested. The maximum number of isolates exhibited resistance to Cefotaxime (17 isolates), Ampicillin (16 isolates), Penicillin (16 isolates), Chloramphenicol (15 isolates), and Carbenicillin (13 isolates). All isolates tested were susceptible to the antibiotics - Kanamycin, Tobramycin, and Rifampicin (**Fig. 4.8**).

4.4. Discussion:

Sediments act as an efficient sink for elements that are stripped off from particle surfaces during stream/fjord water column transport (Herbert et al., 2020), hence supporting our observation of higher trace metal concentrations noted in the sediments as compared to the water samples from terrestrial and fjord systems. The trace metal values observed in our study were comparable to the previous reports from Arctic terrestrial and fjord sediments and waters (**Table 4.3(a)** and **(b)**) with exceptions noted for the higher values of Ni (up to 9.9 µg/Kg), Mn (up to 10.14µg/Kg**)**, Zn (up to 483.7µg/Kg), and Cd (up to 1.16 µg/Kg) in the Kongsfjorden waters as compared to the marine Arctic waters.

We could note that the concentrations of elements like Ni, Zn, and Mn were higher in the terrestrial sediments suggesting the possible terrestrial source of these elements into the fjord waters. The higher concentrations of Ni, Co, Zn, and Mn noted from the terrestrial sediments (**Table 4.2**) can be linked to the abundance of soluble rocks such as carbonates and sulfides in the glacierized areas and the contact of water with glacial debris causing the enrichment of elements (Dragon and Marciniak, 2010). Meltwater streams also allow for the transformation of material from glacial discharge through oxidation of detrital sulphide minerals, thereby generating more reactive particulate trace element species through physical, chemical, and biological weathering (Herbert et al., 2020). Close associations between Co, Ni, and Mn have been well-described in high latitude and marine settings, and likely occur because Co and Ni adsorb onto or incorporate into Mn - OHO (Manceau et al., 1992; Meinhardt et al., 2016). Łokas et al. (2016) has reported the role of atmospheric circulation transporting metals from global and local sources; marine aerosols and precipitation as major suppliers of trace metals in the Arctic glacier-associated and terrestrial environments. Similarly, the higher concentrations of Hg and Pb towards the outer fjord were reported by Lu et al. (2013) suggesting their long-range transport through Atlantic waters into the fjord.

From the metal enrichment experiment, we could observe that the viable bacterial counts varied significantly with the different metals tested ($p < 0.05$). The decreasing order of toxicity of metals based on the viable counts from each of the amendments for

the different samples is Hg > Zn > Co > Cd > Pb > Mn > Ni (**Table 4.4**). It was also evident that the Kongsfjorden samples yielded higher total retrievable bacterial counts as compared to the glacier snout and foreland samples suggesting the presence of more resistant bacterial members in the fjord. The two-way ANOVA also indicated significant differences ($p < 0.05$) in the viable counts of KNS - VB and KNS - BR samples while VB-BR samples were not significantly different in terms of their viable counts. This gives a clear distinction between the terrestrial and fjord locations in terms of their bacterial response to metal amendments.

Another interesting observation is that, unlike the significant variation observed between the water and sediment samples (sample type) based on the background metal concentrations, there was no significant variation in the viable counts between terrestrial sediment - terrestrial waters and fjord sediment - fjord waters. An only significant difference ($p < 0.05$) was noted between the terrestrial and fjord samples. This suggests that irrespective of the higher metal concentrations in the sediment samples as compared to the water samples, sampling location (terrestrial Vs fjord) seem to have a stronger influence on the total retrievable bacterial counts for different metal amendments. This was also supported by the nMDS plot **Fig. 4.3**, wherein VB, BR and KNS formed distinct clusters based on the total retrievable count data from the different metal amendments.

The diversity of cultivable bacteria identified in this study was biased toward Gram-negative strains, mostly belonging to the class γ-proteobacteria (45% of the total) followed by the dominance of α-proteobacteria (19.8%). Class Actinobacteria comprising of gram-positive members accounted for about 15.8% of the total retrievable isolates. Møller et al. (2014) in their study on *merA* gene diversity from the Arctic have reported that the dominant mercury resistant bacterial fraction from freshwater environments in Arctic belonged to γ-proteobacteria and Actinobacteria. Also, a recent study by Rajeev et al. (2021) from coastal Indian sediments has reported the abundance of γ-proteobacteria and Actinobacteria in the heavy metal contaminated areas.

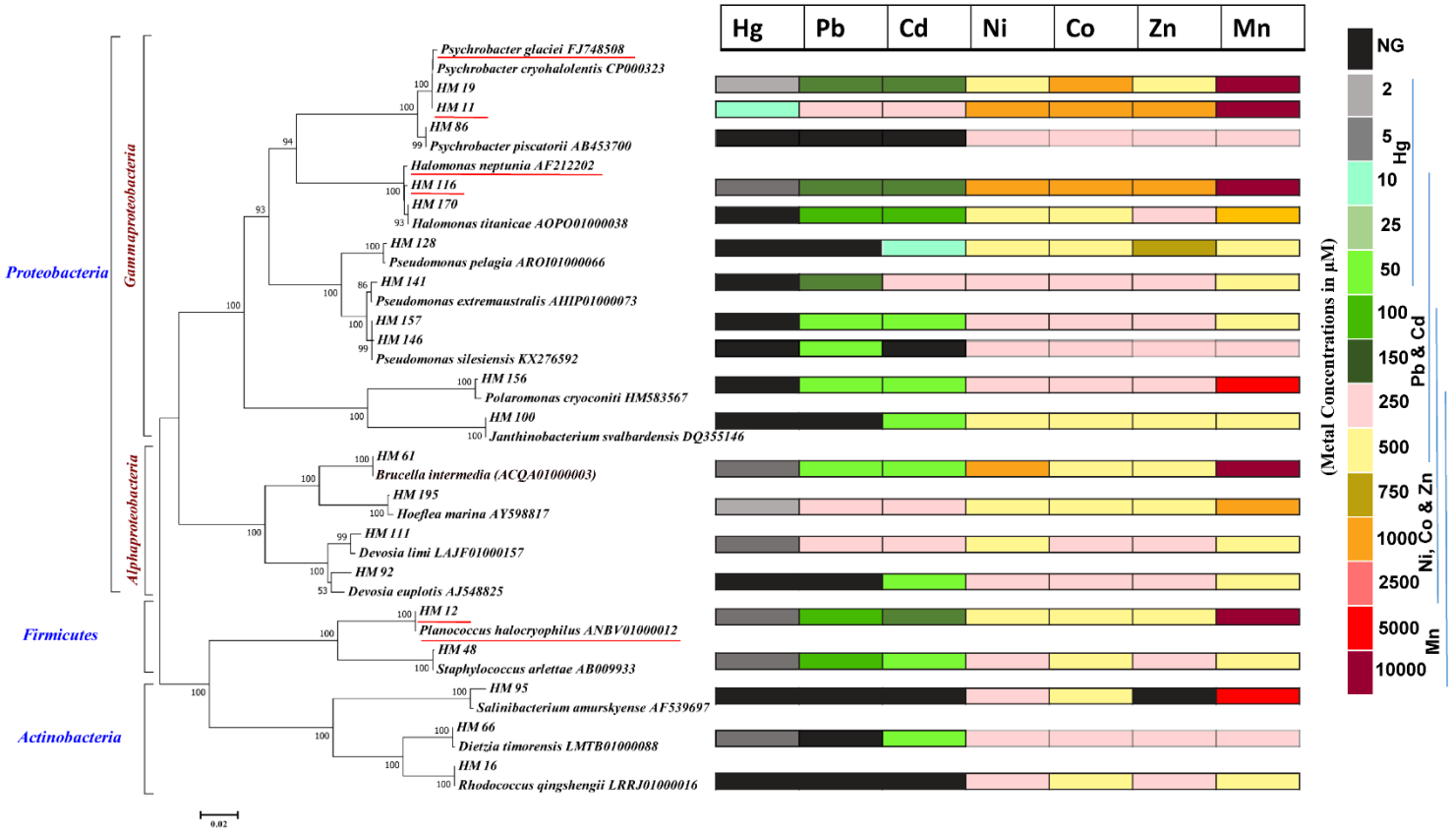


Fig. 4.7. Phylogenetic tree of selected metal tolerant isolates with their levels of metal tolerance expressed as a heat map.

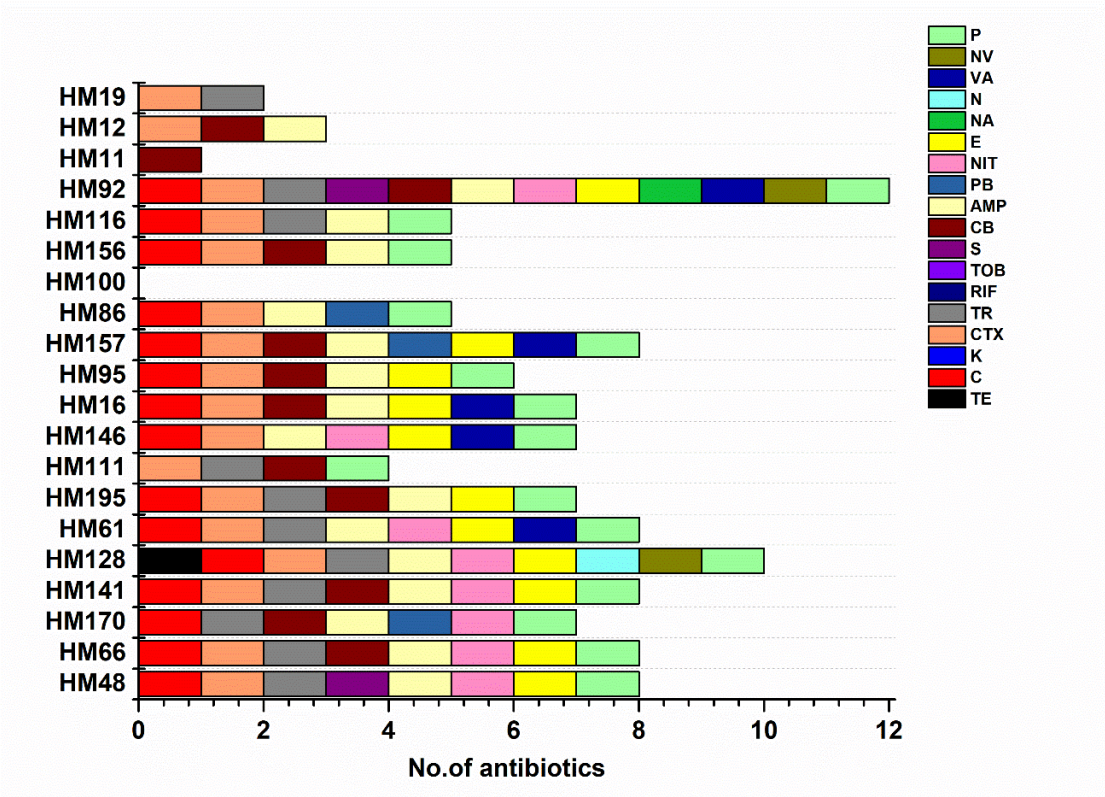


Fig. 4.8. Response of each of the 20 selected bacterial isolates towards 18 different antibiotics tested.

Species belonging to the genus *Pseudomonas* noted from all the sampling locations and all metal amendments in our study have been previously reported for their various inherent metal resistance properties (Orellana-Saez et al., 2019; Møller et al., 2014). Also, the metal removal potential by exopolysaccharide (EPS) production in psychrotolerant Arctic glacier fore-field soil bacterium *Pseudomonas* sp. was reported by Sathiyanarayanan et al. (2016). The glacier-specific species *Janthinobacterium* and *Polaromonas* obtained in our study were known for their association with the cold glacier environments of the Arctic (Thomas et al., 2020; Thomas et al., 2021; Ciok et al., 2018; Avguštin et al., 2013). The presence of Polar bacterial strains belonging to *Pseudomonas* and *Janthinobacterium* sp. having multiple resistance to antibiotics and heavy metals was reported by Tomova et al. (2015). Similarly, a detailed study on the plasmids of *Polaromonas* sp. recovered from both the Poles have revealed the presence

of potential genes involved in the transport of metal ions suggesting their response to the raised levels of heavy metals in Polar regions (Ciok et al., 2018). Among the fjord-specific metal tolerant bacterial isolates, *Salinibacterium, Halomonas, Psychrobacter,* and *Planococcus* were abundant. Members of the *Psychrobacter* genus are versatile and have been isolated from different low-temperature environments including Antarctica and Arctic (Romaniuk et al., 2018; Zeng et al., 2016). The bioremediation potential of marine psychrotolerant *Psychrobacter* species has been reported by Abd-Elnaby et al. (2016) and Romaniuk et al. (2018). The presence of different genes associated with detoxification of heavy metal ions and aromatic hydrocarbons was reported in the cold-tolerant *Planococcus* bacterial genome which was isolated from Antarctica (Jung et al., 2018).

Similarly, there are reports on the heavy metal tolerance potential of *Halomonas* species from non-Polar environments (Xu et al., 2013; Sánchez Porro et al., 2013). *Halomonas neptunia* was reported from deep-sea hydrothermal-vent habitats with dynamic nutrient regimes and fluctuating heavy-metal concentrations (Kaye et al., 2004). Several genes related to the corrosion of metals were reported in the genome of *Halomonas titanicae* explaining its presence on metal surfaces from ships, and its occurrence in contaminated harbors around the world (Sánchez Porro et al., 2013). The bacterial species belonging to *Brucella intermedia* (*Ochrobactum intermedium)* was isolated from both terrestrial and fjord waters in our study and previously reported for their potential to tolerate heavy metal lead and for their metal remediation mechanism by reduction (Waranusantigul et al., 2011 and Mejias Carpio et al., 2018).

While testing for the presence of metal resistant genetic determinants, we could observe the presence of *merA*, *czcA*, *czcC,* and *czcD* genes in the metal tolerant bacterial fraction from the terrestrial and fjord samples suggesting that metal resistant genes (MRGs) are prevalent even in environments with limited anthropogenic influence (**Fig. 4.5** and **4.6**). The presence of *merA* genes occurring frequently in the pristine Polar Regions was reported by Møller et al. (2014) and Mindlin et al. (2016). The presence of all four identified genes was noted in the species closely related to *Pseudomonas silesiensis.*

The internal membrane *czcA* efflux cation-antiporter system along with the CadA enzyme was previously identified in Antarctic Psychrotolerant strain *Pseudomonas* sp. (Orellana-Saez et al., 2019). Similarly, the presence of mercuric reductase gene *merA* was reported from *Pseudomonas sp.* isolated from Arctic snow, freshwater, and sea-ice brine samples by Møller et al. (2014). These studies are indicative that inherent resistant properties towards metals like Cd, Hg, etc are present in the genus *Pseudomonas* from cold Polar Regions. *merA* gene sequences obtained in the study showed maximum similarity to the mercuric reductase gene (both plasmid- and chromosome-associated) from *Halomonas, Pseudomonas*, and *Burkholderia* species. Similarly, the *czcA* gene sequences identified in the study exhibited the highest similarity percentage to chromosome-associated cobalt, zinc, cadmium protein from *Pseudomonas yamanoram* and *Pseudomonas mandeli*, chromosome-associated cation transporter protein reported from *Pseudomonas alcaliphila* and *P. frederiksbergensis*, and chromosome-associated efflux RND transporter permease subunit from *Janthinobacterium* species. All the *czcC* gene sequences showed the highest percentage similarity to the chromosome 2-associated heavy metal efflux RND transporter czcC gene from *Cupriavidus gilardii* while all the *czcD* gene sequences showed 100% similarity percentage to chromosome 2-associated cation transporter from *Cupriavidus gilardii* indicating the specificity of primer pairs used. Therefore, it was noted that the gene sequences for *merA* and *czcA* genes, differed for different bacterial species while for *czcC* and *czcD* genes, conserved gene sequences were present in phylogenetically different species.

For the primer pairs specific to *nccA, smt, and mnx* genes, no PCR products were obtained for any of the tested isolates. Similarly, among the 20 selected metal tolerant isolates only a few species like *Halomonas neptunia* (HM116), *Halomonas titanicae* (HM170), *Pseudomonas extremaustralis* (HM141), *Pseudomonas silesiensis* (HM157, HM146), *Devosia limi* (HM111), and *Dietzia timorensis* (HM66) possessed the tested metal resistant gene elements, suggesting that they are either missing in the genomes of the other studied bacteria, or the observed resistance phenotypes might be "nonspecific" or driven by other systems, for e.g., altered cell envelope permeability or biofilm formation (Wales and Davies, 2015).

The 20 short-listed bacterial isolates based on their multi-metal tolerance potential belonging to the genera *Psychrobacter, Halomonas, Planococcus, Pseudomonas, Brucella, Hoeflea, Devosia, Salinibacterium, Staphylococcus, Rhodococcus* and *Dietzia* were tested for their MIC towards different metals. As per Nieto et al. (1987) and Abou- Shanab et al. (2007), a bacterial strain was recognized as resistant to a particular heavy metal ion when it was capable of growing at the following ion concentrations: (i) 1 mM Cd(II), Co(II), Ni(II), Zn(II), and (iii) 0.1 mM Hg(II). In our study, the highest concentrations of metals which allowed the bacterial growth were Hg-10µM, Pb, and Cd- 250µM, Ni, Co, Zn- 1mM, and Mn-10mM. From the results, it was observed that Hg is more toxic since the highest concentration that the bacteria could tolerate was 10µM as compared to the other metals. This is in agreement with the previous works reported (Neethu et al. 2015, Romaniuk et al. 2018). Although the levels of metal tolerance of bacterial isolates in our study were much lower as compared to the values reported by Neethu et al. (2015) from Kongsfjorden, the highest tolerable concentrations were much higher as compared to the background metal concentrations observed in the study. The species belonging to the genera *Psychrobacter, Planococcus,* and *Halomonas* sp. exhibited the highest MIC levels for different metals and these species are reported for their metal resistance previously (Abd-Elnaby et al., 2016; Romaniuk et al., 2018; Jung et al., 2018; Sánchez Porro et al., 2013; Kaye et al., 2004).

The test of toxicity of heavy metals investigated here could be useful in the evaluation of metal toxicity and their impact on microbial communities in the Arctic regions. Our study showed that the glacier and the fjord system have different metal tolerant bacterial species exhibiting varying levels of tolerance to the metals tested. Also, the resistance phenotypes obtained in the study were not being influenced by their phylogenetic relatedness. Moreover, the co-resistance (resistance towards at least two metals) phenotypes among the metal tolerant bacterial fraction suggesting that this phenomenon is not restricted to bacteria originating from the heavily contaminated regions but even in low human-impacted Arctic regions, where such resistance traits enable the bacteria to cope with the occurrence of pollutants (Giudice et al., 2013; Romaniuk et al., 2018).

Some evidence strongly suggests that metal tolerance and antibiotic resistance are closely associated with each other in bacteria (Timoney et al., 1978). Therefore, we have assessed the antibiotic resistance properties of the 20 selected metal tolerant bacterial isolates. Antibiotics used in this particular study represent several groups such as the β-lactams, penicillins, cephalosporins, tetracyclines, glycopeptides, aminoglycosides, aminoglycerides, macrolides, lincosamides, aminocyclitols, quinolones, etc. The multiple antibiotic resistance noted for most of the isolates may indicate the co-occurrence of genes for resistance to antibiotics and metals within their genomes or on their mobile genetic elements which need to be studied in detail. The presence of R-plasmid-containing genes as a possible reason for bacterial resistance towards both antibiotics and heavy metals was reported by Timoney et al. (1978), Calomiris et al. (1984), Saikia et al. (2008), and Neethu et al. (2016). It is important to remark that these metal tolerant bacteria retrieved from the Arctic can exhibit antibiotic resistance, especially towards the β-lactams and penicillin. This is an indication that the local microbiota could harbor naturally occurring metal resistant genes and antibiotic-resistant genes which could further be potentially transmitted among other bacterial members in the system (Jara et al., 2020) via migratory birds or animals which could act as a potential carrier for the dissemination of such resistant elements (Prakash et al., 2022; Hatha et al., 2021).

To our knowledge, this is the first study giving a detailed description of the metal tolerant bacterial fraction associated with a glacio-marine system in Ny-Ålesund, Arctic. The study indicates the potential of Arctic bacteria to tolerate metals that are of ecological significance to the Arctic (Hg, Cd, Pb, Ni, Co, Zn, and Mn). 90% of the multi-metal resistant isolates also exhibited multi-drug resistance potential which needs to be looked into. Further, we need to understand how the metal concentrations impact these Arctic bacterial physiology/metabolism and what the bacterial responses towards these metals are. This will be discussed in detail in **Chapter 5**.

Chapter 5.

Understanding the metal-bacterial interactions in selected Arctic isolates

Chapter. 5

Understanding the metal-bacterial interactions in selected Arctic isolates

5.1. Introduction

In any environment, there exists an interaction between the microbial community and the metals and minerals present there. Microbes interact with metals and minerals altering their physical and chemical state, while metals and minerals can affect microbial growth, activity, and survival (Gadd, 2010). Metals like zinc, copper, nickel, iron, etc. are defined as essential micronutrients for microbial growth since they play a crucial role in biological processes inside the cells, such as redox reactions, osmotic regulation, acting as coenzymes, etc (Nies, 1999; Bruins et al., 2000). Contrarily, other metals, such as cadmium, mercury, and lead, are nonessential and are harmful to physiological functions (Bruins et al., 2000). Generally, the toxic effect of the metal is indicated by the interaction between the ionic forms of metals with ligands, such as sulfhydryl groups of microbial enzymes, thus inhibiting their enzymatic activities (Olaniran et al., 2013). When present in concentrations exceeding the threshold levels required by the bacterial cells, metals can affect the bacterial morphology, physiology, and metabolism through functional disturbance, protein denaturation, or destroying cell membrane integrity (Haferburg and Kothe, 2007). However, interaction with metals can also at times induce resistant properties in bacteria like antibiotics/antimicrobial resistance (Neethu et al., 2015).

It is well studied that the presence of excess concentrations of metals in the environment, has led to the development of ingenious mechanisms of metal tolerance in bacteria such as accumulation, metal efflux, detoxification, etc. to counteract the stress induced by the toxic levels of metals (Habi and Daba, 2009; Laganà et al., 2018). Some bacterial resistance mechanisms tend to be specific for a particular metal while other mechanisms are rather non-specific conferring resistance to a variety of metals.

Therefore, it is indicative that the bacterial population present in any environment may directly influence the fate of metals and cause their transformation into less toxic states. This could further decrease the metal contamination levels in the environment. Hence, metal-resistant bacteria can be considered as potential bio-indicators of pollution. Furthermore, bacterial metal tolerance was proposed as an ecological indicator of potential toxicity to all other forms of life in several environments (AMAP, 2011; Das et al., 2009; Ellis et al., 2001; Papale et al., 2018).

As observed in **Chapter 4**, a specific metal tolerant bacterial population was selected in our study when Arctic glacio-marine water and sediment samples were subjected to different metal enrichments. The screening of the metal tolerant bacterial fraction lead to the identification of a few potential multi-metal tolerant bacterial species like HM11 closely related to *Psychrobacter glaciei*, (99.86%), HM116 closely related to *Halomonas neptunia* (99.57%), and HM12, closely related to *Planococcus halocryophilus* (100%). The fact that all three selected isolates belong to the Kongsfjorden sedimentary environment can be linked to the higher metal concentrations observed in the fjord sediments as compared to the other Arctic samples. This also highlights the adaptive strategies of fjord sediment isolates to adjust to the rapidly changing system of Kongsfjorden which receives materials through the Atlantic water intrusion as well as through the glacier melting. Thus, in this chapter we focused on the metal-bacterial interactions using these three selected bacterial isolates, to understand:

a) The effect of metals on bacterial enzyme production, carbon substrate utilization, pigment production, and antibiotic resistance.

b) The metal bioaccumulation properties of bacteria for metal mitigation.

5.2. Methodology

The three bacterial cultures HM11, HM12, and HM116 were maintained in a low concentration Nutrient broth media ($1/100^{th}$ strength, M002, HiMedia, India, referred to as dNB) at 15 °C incubation. The fully grown bacterial cultures in the dNB were centrifuged (5000 rpm, 15 min) and the resulting pellet was washed 3 times with 0.9% saline. The bacterial inoculum thus obtained was fixed to an optical density (OD) of 0.5 at 600nm and the corresponding number of bacterial cells were measured using epifluorescence microscopy by staining the bacterial cells with DAPI (20 µl of 1 µg ml^{-1} working solution per ml). In brief, the DAPI stained bacterial cultures were filtered onto 0.22 µm Nucleopore Track-etched polycarbonate black membrane filters (Whatman, Maidstone, UK) and counted using Leica DM6 B microscope (Leica Microsystems, Wetzlar, Germany) as described in **Chapter 3, Section 3a.2.4**. Further, the inoculum of cells were transferred to dNB with different metals in varying concentration. The metals used for the study included Hg, Cd, Pb, Ni, Co, Zn, and, Mn in the form of their analytical grade salts - $HgCl_2$, $CdCl_2.H_2O$, $ZnCl_2$, $Pb(NO_3)_2$, $NiCl_2.6H_2O$, $CoCl_2.6H_2O$, and $MnCl_2.4H_2O$ respectively (Merck). The concentration of metals used are as follows:

Table 5.1.

Metals and their concentrations (in µM) used for the study.

Metals Used	**Concentration chosen (µM)**
Hg	2, 5 and 10
Pb	50, 100 and 150
Cd	50, 100 and 150
Ni	100, 250 and 500
Co	100, 250 and 500
Zn	100, 250 and 500
Mn	250, 500 and 1000
C1 (Hg+Pb+Cd)	Hg (2), Pb and Cd (10)
C2 (Ni+Co+Zn+Mn)	100 µM each

The fully grown bacterial cultures (14th day) with and without metal amendments were further subjected to the following assays.

5.2.1. Impact of metals on bacterial enzyme production

Amylase, gelatinase, cellulase, protease, lipase, catalase, and oxidase production were tested for all 3 isolates which were initially grown in dNB with and without metal amendments. The isolates grown in the broth medium were further subcultured on to dNB agar plates to be used for the enzyme assays. Amylase, gelatinase, and cellulase enzyme assays were done by spot inoculating the cultures on Modified Basal Media (MBM, modified by Bhosle, 1981) plates with 0.5% starch and Carboxy Methyl Cellulose (CMC), 1% gelatin (with and without the metal amendments). All the enzyme substrates were procured from Himedia, India. The media composition for MBM is as follows: NaCl - 25g, KCl - 0.75g, $CaCl_2$ - 0.2g, $MgSO_4.7H_2O$ - 7g, NH_4Cl - 0.5g, Yeast extract - 2g, Peptone - 0.3g, K_2HPO_4 (10%) - 7mL and KH_2PO_4 (10%) - 3mL in 1L (pH - 7). For lipase assay, Tributyrin Agar Media (TBA, M157-Himedia, India) with and without metal amendments was used. The lipid substrate used for the TBA was 1% tributyrin (Gupta et al., 2003). Similarly, skim milk agar media (M1213, Himedia, India), with and without metal amendments was used for the protease test. The plates were incubated for one week at 15 °C. Amylase production was tested by flooding the plates with Gram's Iodine solution (Iodine - 1gm, KI - 1gm in 100mL MilliQ water) (Khandeparker et al., 2011). Cellulase production was tested by flooding the plates with 1% Congo red solution for 15 min and then washing the plates multiple times with 1M NaCl (Khanderparkar et al., 2011). Gelatinase activity was demonstrated, by flooding the plates with acid mercuric chloride solution ($HgCl_2$ - 15 g, Con. HCl - 20ml, MilliQ water - 80mL) (Balan et al., 2012). The appearance of a clearing zone around the colonies was noticed as the positive result for all enzyme assays.

For the oxidase test, oxidase discs that were impregnated with N, N-dimethyl-p-phenylenediamine oxalate, ascorbic acid and α-naphthol were used (Himedia, DD018). Oxidase reaction was carried out by touching and spreading the bacterial cultures on the oxidase disc. The reaction was observed within 5-10 seconds as a colour change to deep blue or purple. The oxidase discs for the test were procured from Himedia (https://himedialabs.com/TD/DD018). For the catalase test, the bacterial cultures in the

broth media were added to a drop of water taken on a microscope slide followed by mixing with 3% H_2O_2 solution. The production of the rapid brisk effervescence of oxygen was taken as a positive result for catalase (http://microbeonline.com/catalase-test-principle-uses-procedure-results/).

5.2.2. Impact of metals on antibiotic resistance in bacteria

The antibiotic susceptibility test for the three selected isolates was carried out by the Kirby-Bauer method (Bauer et al., 1966) on the Nutrient agar ($1/100^{th}$ strength, Hi Media, India). Selected isolates which were enriched in nutrient broth - with and without metals, were aseptically swabbed onto agar plates. The antibiotics tested were ampicillin (10 mcg), amoxycillin (30 mcg), nalidixic acid (30 mcg), tetracycline (30 mcg), neomycin (30 mcg), novobiocin (30 mcg), cefotaxime (30 mcg), penicillin-G (10 mcg), polymyxin-B (30 mcg), tobramycin (30 mcg), chloramphenicol (30 mcg), vancomycin (30 mcg), kanamycin (30 mcg), rifampicin (30 mcg), streptomycin (10 mcg), tetracycline (30 mcg), carbenicillin (100 mcg), and nitrofurantoin (300 mcg) (Himedia). The results were assessed by measuring the zone of inhibition after a period of 5 days at 15°C incubation.

5.2.3. Impact of metals on bacterial carbon substrate utilization

The carbon substrate utilization experiment was carried out as per the protocol detailed in **Chapter 3, section 3(b).2.7**. Substrates tested included D-cellobiose, sucrose, D-mannitol, D-Trehalose, D-lactose, D-glucose, D-fructose, D-galactose, and N-acetyl-D-glucosamine. These substrates were filter-sterilized before use. Each of the substrates was added to a Minimal media (detailed in **Chapter 3, section 3(b).2.7)** to produce a final concentration of 50mg/L (Freese et al. 2010), followed by amendments with different metals in varying concentrations as described in **Table 5.1**. There were also control media with only the substrate in the minimal media (without metals) and only the minimal media without any substrates and any metals. The experiment was tested at 15°C incubation. Absorbance (OD 600nm) was measured for 14 days at regular intervals of 0, 7, and 14 days.

5.2.4. Impact of metals on bacterial pigment production

Among the three isolates, HM12 (*Planococcus halocryophilus*) was orange pigmented and therefore tested to understand the impact of metals on their pigment production properties. For the assay, HM12 culture was grown for 7 days as well as for 14 days of incubation in a low concentration Nutrient Media (dNB), referred to as the positive control and in dNB with different metal amendments (**Table 5.1**) were used (15 ml broth). The broth cultures were centrifuged at 5000 rpm for 10 minutes at 4 °C and the resultant pellet was mixed with 2ml of 90% acetone. The cell suspension was then homogenized by vortexing thoroughly followed by sonication using an ultrasonic homogenizer (30s burst at 30W for 5 times in dark). The extraction of pigments was allowed for 12 h overnight at - 20 °C. After the extraction, the supernatant was collected by centrifugation (5000 rpm, 10 min at 4 °C) (Singh et al., 2017) and subjected to spectral analysis using a multi-mode plate reader (Synergy 2-multi mode microplate reader, BioTek Instruments, USA).

5.2.5. Metal removal efficiency of the bacterial isolates - Experiment set-up

For assessing the metal removal efficiency of bacteria, the selected 3 bacterial isolates (HM11, HM12, and HM116) were grown in dNB spiked with different metals. Positive (cultures grown in dNB without any metals) and negative controls (dNB without bacteria and any metal amendments, dNB without bacteria but with different metal amendments) were also maintained in triplicates for the experiment. The metal concentrations chosen for the study are given in **Table 5.2** and the values were comparable to the environmental metal concentrations.

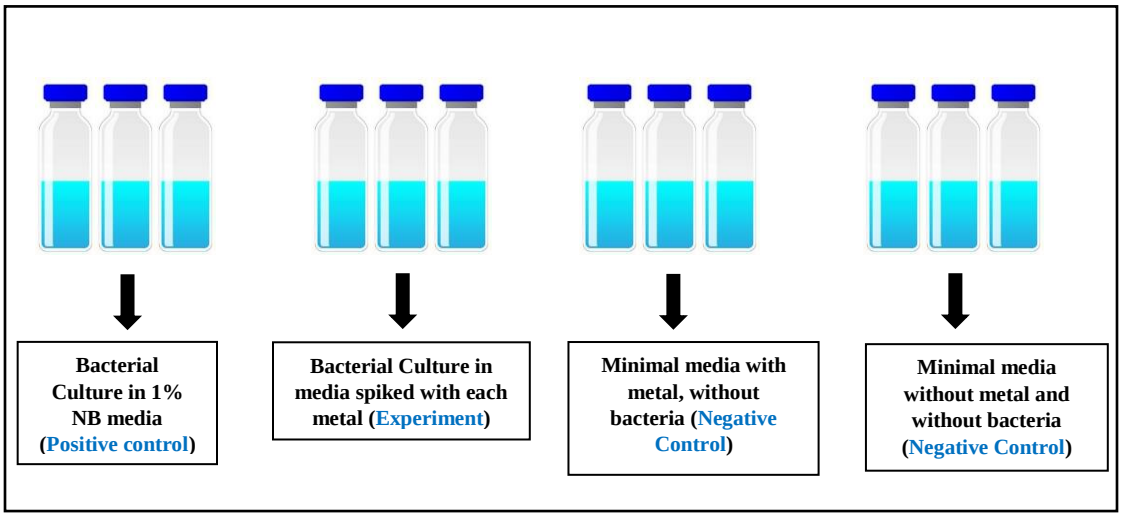


Fig. 5.1. Experimental setup for bioaccumulation assay.

Table 5.2.

Metals and their concentrations used (in ppm) for the bioaccumulation assay.

Metals used	**Concentration (in ppm)**	**Equivalent concentration in µM**
Pb ($Pb(NO_3)_2$)	20	96.5
Cd ($CdCl_2.H_2O$)	10	88.96
Ni ($NiCl_2.6H_2O$)	25	425.98
Co ($CoCl_2.6H_2O$)	25	424.18
Zn ($ZnCl_2$)	25	381.55
Mn ($MnCl_2.4H_2O$)	50	909.55

Bacterial cultures for the experiment (15ml each) were incubated for 14 days at 15 °C at 120 rpm shaking condition in an orbital shaker incubator (Optic Ivymen system, Biotech, Spain). All experimental samples were maintained in triplicates. To detect the metals bound to the bacterial cells, heavy metal analysis using Inductively coupled plasma-optical emission spectrometry (ICP-OES) was carried out as per Chaudhary et al. (2017) with few modifications. In brief, the bacterial cultures (15ml) at each time interval (7^{th} day and 14^{th} day), were centrifuged at 5000 rpm for 10 minutes. The cell

pellets thus obtained were washed 3 times with 0.9% NaCl, dried overnight in a hot-air oven at 60 °C, acidified by the addition of 5ml Con. HNO_3 (Suprapur, Merck Millipore, Germany) and kept for open digestion in Teflon beakers at 120 °C for 24 hours in a clean-room fume hood. The digested pellets were reconstituted to 25ml by the addition of 2% HNO_3 (Suprapur) in 25ml standard flasks. The initial day (0^{th} day) samples (which includes negative controls and metal amended broths) were also digested following the same procedure. The 0^{th}- day samples and the digested pellets for the 7^{th} and 14^{th} - day incubation were analyzed using the ICP-OES (Thermo scientific, iCAP 7000 series, UK) for Pb, Cd, Ni, Co, Zn, and Mn measurements. All the centrifuge tubes (15ml) used in the study were acid cleaned (using 2% Con. HNO_3 (Suprapur)) and dried before transferring the samples.

The metal removal (%) for each bacterial isolate was calculated as per the equation:

$$\textbf{Metal removal (\%) = [Ci – Cf/Ci]*100}$$

where; Ci and Cf are the initial and final concentrations of metal, respectively (Ameen et al., 2020).

The bacterial growth for each of the isolates HM11, HM12, and HM116 was also measured in the experiment at regular time intervals of 0, 7, and 14 days for the positive control and the cultures grown in different metal amendments, to determine whether there is any correlation between the bacterial growth performance and heavy metal removal efficiency (%).

5.2.6. Statistical analysis

One-way Analysis of Variance (ANOVA) using ORIGIN, v.9 software was carried out to determine significant differences in the bacterial carbon substrate utilization, and pigment production for cultures grown in different metals in comparison to the positive controls for each of the isolate. Similarly, to assess the significant differences in metal removal efficiency between the bacterial isolates and also differences in removal efficiency for different metals, one-way ANOVA was carried out (ORIGIN v.9). The significance level was set at a P value < 0.05 as significant. Pearson correlation analysis

was carried out using ORIGIN, v.9 software to determine the interactions between bacterial growth in terms of cell count and their heavy metal removal efficiency.

5.3. Results:

5.3.1. Impact of metals on bacterial enzyme production

We could observe that the three bacterial cultures HM11 (*Psychrobacter glaciei*), HM12 (*Planococcus halocryophilus*), and HM116 (*Halomonas neptunia*) exhibited varying responses with respect to enzyme production both in the presence and absence of metals. HM11 which was grown in dNB alone (positive control) was positive for amylase, gelatinase and protease production. It was observed that metals such as Pb, Cd, Co, and Mn inhibited amylase, gelatinase and protease production at the highest concentrations tested in the study (150 µM for Pb, 100 and 150 µM for Cd, 500 µM for Co, and 1000 µM for Mn) (**Table 5.3**). The lipase production was stimulated for HM11 isolate grown in the presence of Cd, Ni, and Mn which was otherwise not observed for the positive control. For the isolate HM12, the positive control exhibited gelatinase and protease production while for the isolate HM116, the positive control exhibited amylase, gelatinase, protease and lipase production. As seen in **Table 5.3**, for the isolate HM12, protease production was not affected by the metal amendments except for zinc and metal combination C2. Similarly, for the isolate HM116, amylase, gelatinase, protease, and lipase production were unaffected by Pb amendments while all other metal amendments inhibited one or all of the enzyme production properties. We could observe that higher concentrations of all metals have an inhibitory effect on bacterial enzyme production.

Also, there was no enzyme production noted for any of the isolates grown in Zinc amended broth media and in metal combination C2 (Ni+Cd+Zn+Mn).

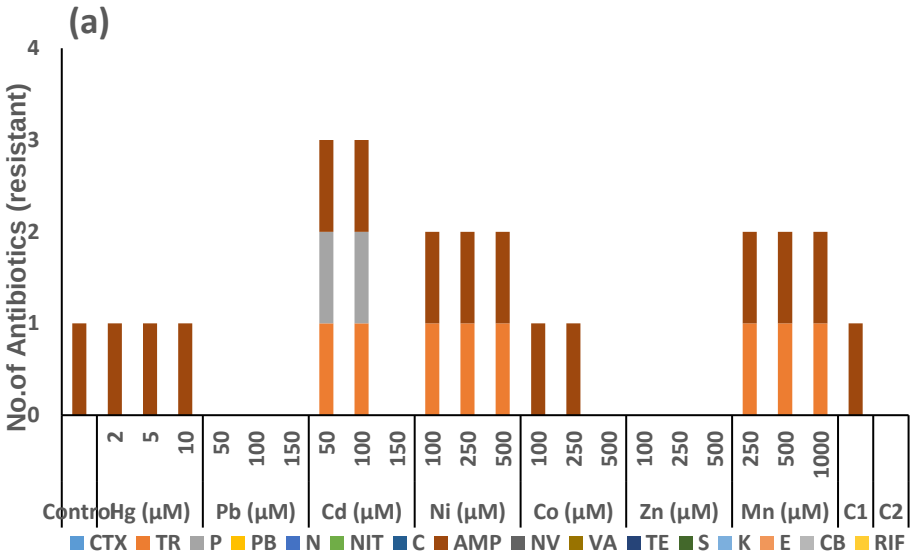
Table 5.3.

Enzyme production potential of HM11, HM12, and HM116 grown in Nutrient Broth (1/100th strength, positive control) and the different metal amendments.

	Control	Hg (µM)			Pb (µM)			Cd (µM)			Ni (µM)			Co (µM)			Zn (µM)			Mn (µM)			C1	C2
		2	5	10	50	100	150	50	100	150	100	250	500	100	250	500	100	250	500	250	500	1000		
HM11 *(Psychrobacter glaciei)*																								
Amylase	+	+	+	+	+	+	-	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-
Gelatinase	+	+	+	+	+	+	-	+	-	-	+	+	+	+	+	-	-	-	-	+	+	-	+	-
Cellulase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Protease	+	+	+	+	+	+	-	+	-	-	+	+	+	+	+	-	-	-	-	+	+	-	+	-
Lipase	-	-	-	-	-	-	-	+	-	-	+	+	+	-	-	-	-	-	-	+	+	-	-	-
Catalase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HM12 *(Planococcus halocryophilus)*																								
Amylase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gelatinase	+	+	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Cellulase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Protease	+	+	+	-	+	+	+	+	-	-	+	+	+	+	+	+	-	-	-	+	+	+	+	-
Lipase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Catalase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HM116 *(Halomonas neptunia)*																								
Amylase	+	-	-	-	+	+	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-
Gelatinase	+	-	-	-	+	+	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-
Cellulase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Protease	+	-	-	-	+	+	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-
Lipase	+	+	+	+	+	+	+	+	-	-	+	+	+	-	-	-	-	-	-	+	+	+	+	-
Catalase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

5.3.2. Impact of metals on bacterial antibiotic sensitivity/resistance response

For the isolate HM11 (*Psychrobacter glaciei*), the positive control (bacteria grown in dNB) exhibited resistance only to Ampicillin while the isolate enriched with different concentrations of Cd (50µM and 75µM), Ni (100µM, 250µM, and 500µM), and Mn (250µM, 500µM, and 1000µM) induced resistance to Trimethoprim along with their resistance to Ampicillin. Similarly, Cd concentrations also induced resistance to the antibiotic Penicillin for the isolate HM11. It was also noted that Pb and Zn amendments and the metal combination C2 inhibited the antibiotic resistance in HM11 bacteria completely (**Fig. 5.2a**). For the isolate HM12 (*Planococcus halocryophilus*), antibiotic resistance properties remain unaffected by the metal enrichments (**Fig. 5.2b**). For the isolate HM116 (*Halomonas neptunia*), the bacteria enriched with different concentrations of $ZnCl_2$ inhibited its resistance towards antibiotics Trimethoprim, Penicillin, and Ampicillin which otherwise was resistant towards these antibiotics in their positive control and other metal enrichments (**Fig. 5.2c**).



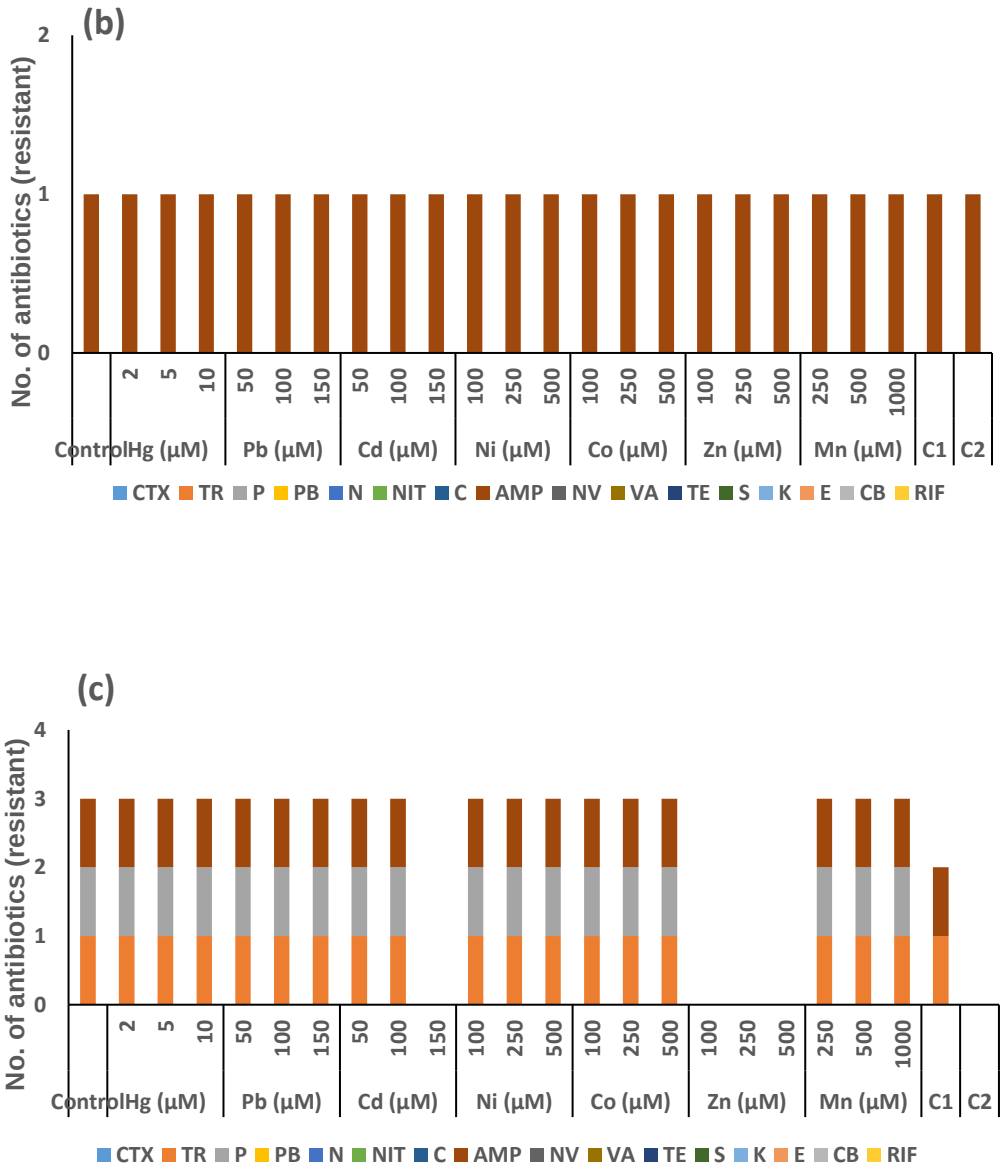
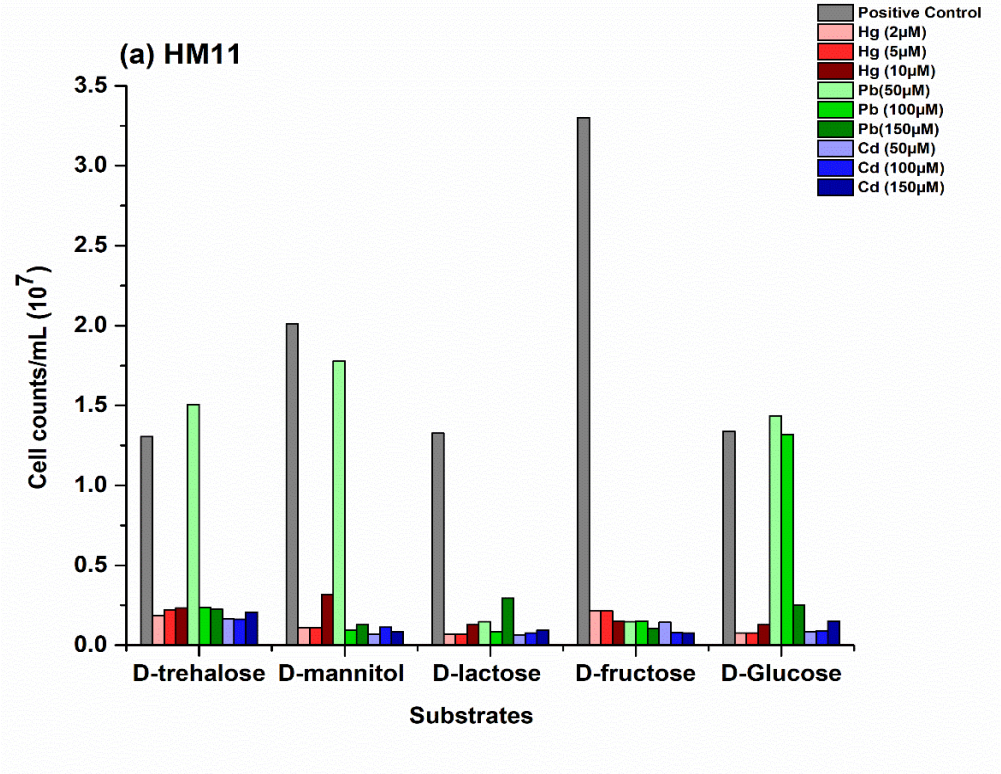


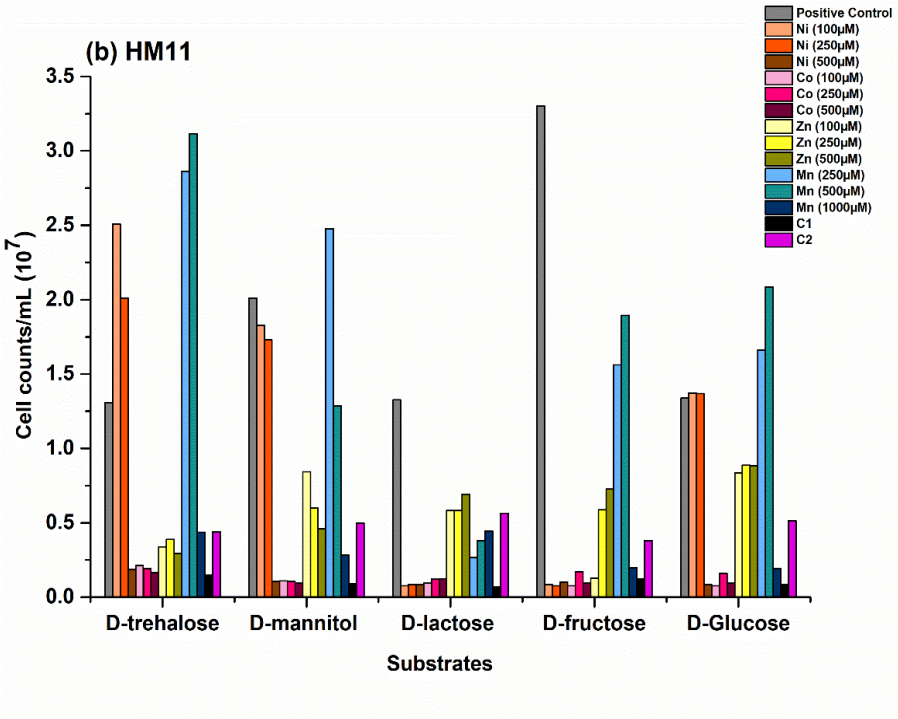
Fig.5.2. Antibiotic resistance seen in isolates **(a)** HM11 (*Psychrobacter glaciei*), **(b)** HM12 (*Planococcus halocryophilus*), and **(c)** HM116 (*Halomonas neptunia*) in the positive control as well as in the presence of different metals in varying concentrations. + indicates the positive control for each isolate (culture grown without any metals).

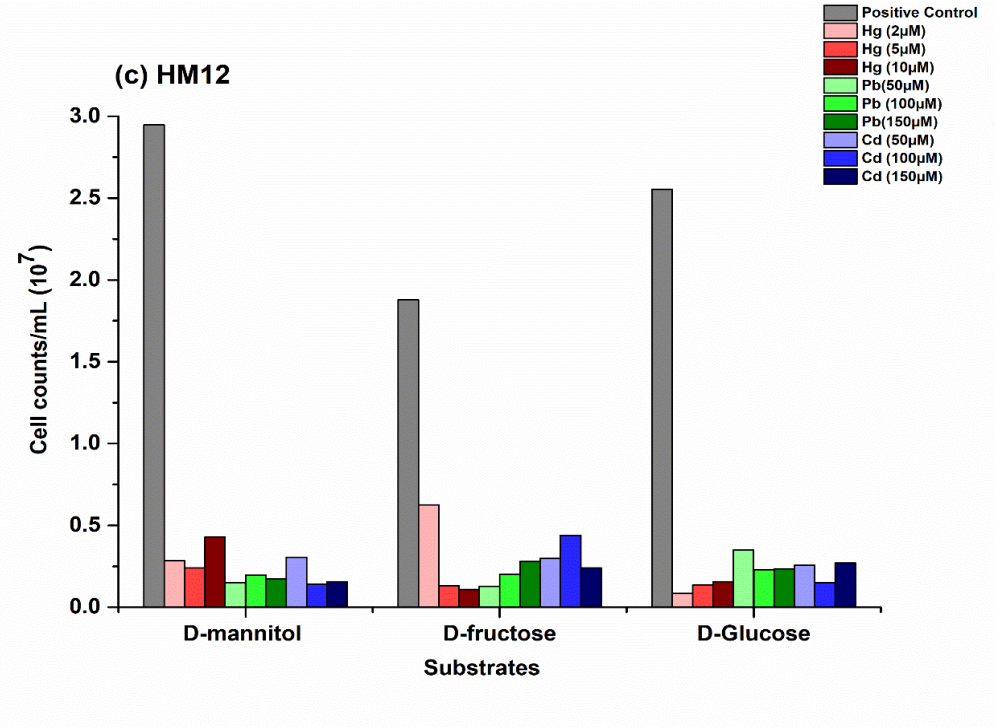
5.3.3. Impact of metals on bacterial carbon substrate utilization

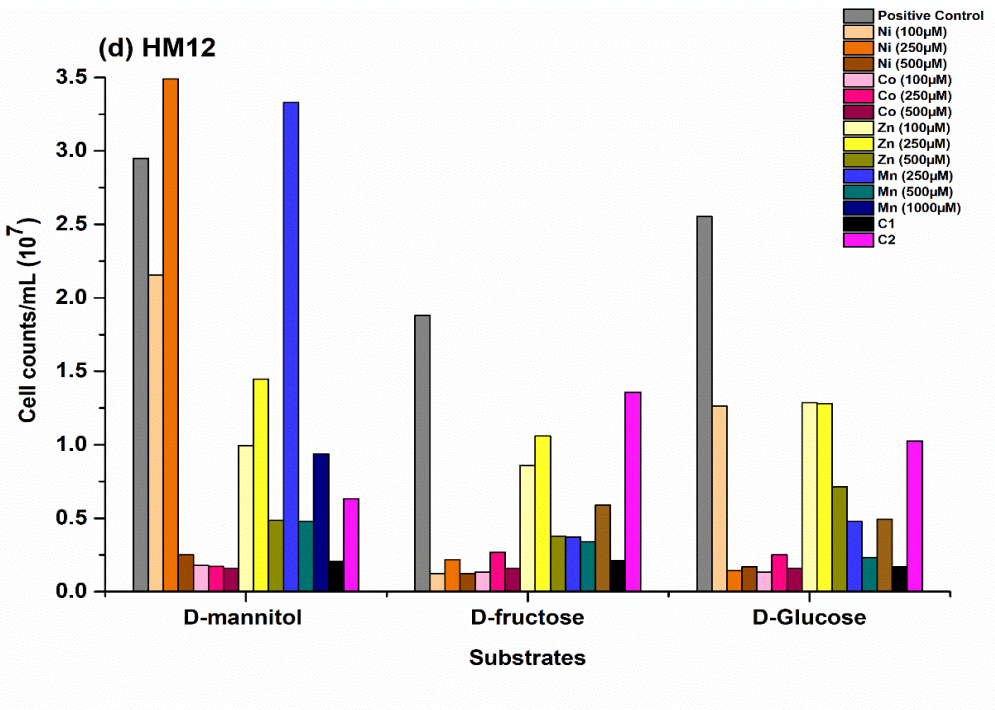
It was observed that isolate HM11 (*Psychrobacter glaciei*) grown in 1% NB was positive for the utilization of substrates such as D-trehalose, D-mannitol, D-lactose, D-glucose, and D-fructose. In the presence of metals such as Hg, Cd, Co, Zn, and metal combinations C1 and C2, the isolate exhibited no growth in any of these substrates while the lower concentrations of metals such as Pb (50 µM), Ni (100 and 250 µM), and Mn (250, 500 µM) enabled bacteria to utilize the substrates D-trehalose, D-mannitol, and D-glucose (**Fig. 5.3 (a) and (b)**). This observation was further supported by the one-way ANOVA, which showed a significant reduction in the bacterial cell count ($p<0.05$) in all amendments of Hg (5, 10 and 25 µM), Cd (50, 100, 250 µM), Co (100, 250, 500 µM), Zn (100, 250, 500 µM), higher concentrations of Pb (100, 250 µM), Ni (500 µM), and Mn (1000 µM), and the C1, C2 combinations in comparison to that of the positive control for the different carbon substrates. However, there was no significant difference in bacterial cell counts between the positive control and the culture grown in lower concentrations of Pb, Ni, and Mn.

For the isolate HM12 (*Planococcus halocryophilus*), the positive control (bacteria in 1% NB) exhibited growth in the presence of D-mannitol, D-glucose, and D-fructose while the presence of metals such as Hg, Pb, Cd, Co, Zn and metal combinations, inhibited their growth in these substrates (**Fig. 5.3 (c) and (d)**). One-way ANOVA yielded a significant reduction in the bacterial cell counts ($p < 0.05$) in all concentrations of Hg, Pb, Cd, Co, and metal combinations C1 and C2 in comparison to that of the positive control, while there was no significant difference in cell counts noted for lower concentrations of Ni (100, 250 µM), Zn (100, 250 µM) and Mn (250 µM) in comparison to that of the positive control. Similarly, for the isolate HM116 (*Halomonas neptunia*), the positive control exhibited growth in the presence of D-trehalose, D-mannitol, D-lactose, D-glucose, D-fructose, D-galactose, and N-acetyl-D-glucosamine (**Fig. 5.3 (e) and (f)**). Except for the higher rate of bacterial growth in the lower Pb concentrations (50, 100 µM), all other metal amendments yielded a significant reduction in growth in different carbon substrates compared to the positive control (one-way ANOVA, $p < 0.05$).


(a) HM11
Positive Control
Hg (2µM)
Hg (5µM)
Hg (10µM)
Pb(50µM)
Pb (100µM)
Pb(150µM)
Cd (50µM)
Cd (100µM)
Cd (150µM)
Cell counts/mL (10^7)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
D-trehalose
D-mannitol
D-lactose
D-fructose
D-Glucose
Substrates


(b) HM11
Positive Control
Ni (100µM)
Ni (250µM)
Ni (500µM)
Co (100µM)
Co (250µM)
Co (500µM)
Zn (100µM)
Zn (250µM)
Zn (500µM)
Mn (250µM)
Mn (500µM)
Mn (1000µM)
C1
C2
Cell counts/mL (10^7)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
D-trehalose
D-mannitol
D-lactose
D-fructose
D-Glucose
Substrates


(c) HM12
Positive Control
Hg (2μM)
Hg (5μM)
Hg (10μM)
Pb(50μM)
Pb (100μM)
Pb(150μM)
Cd (50μM)
Cd (100μM)
Cd (150μM)
Cell counts/mL (10^7)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
D-mannitol
D-fructose
D-Glucose
Substrates


(d) HM12
Positive Control
Ni (100μM)
Ni (250μM)
Ni (500μM)
Co (100μM)
Co (250μM)
Co (500μM)
Zn (100μM)
Zn (250μM)
Zn (500μM)
Mn (250μM)
Mn (500μM)
Mn (1000μM)
C1
C2
Cell counts/mL (10^7)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
D-mannitol
D-fructose
D-Glucose
Substrates

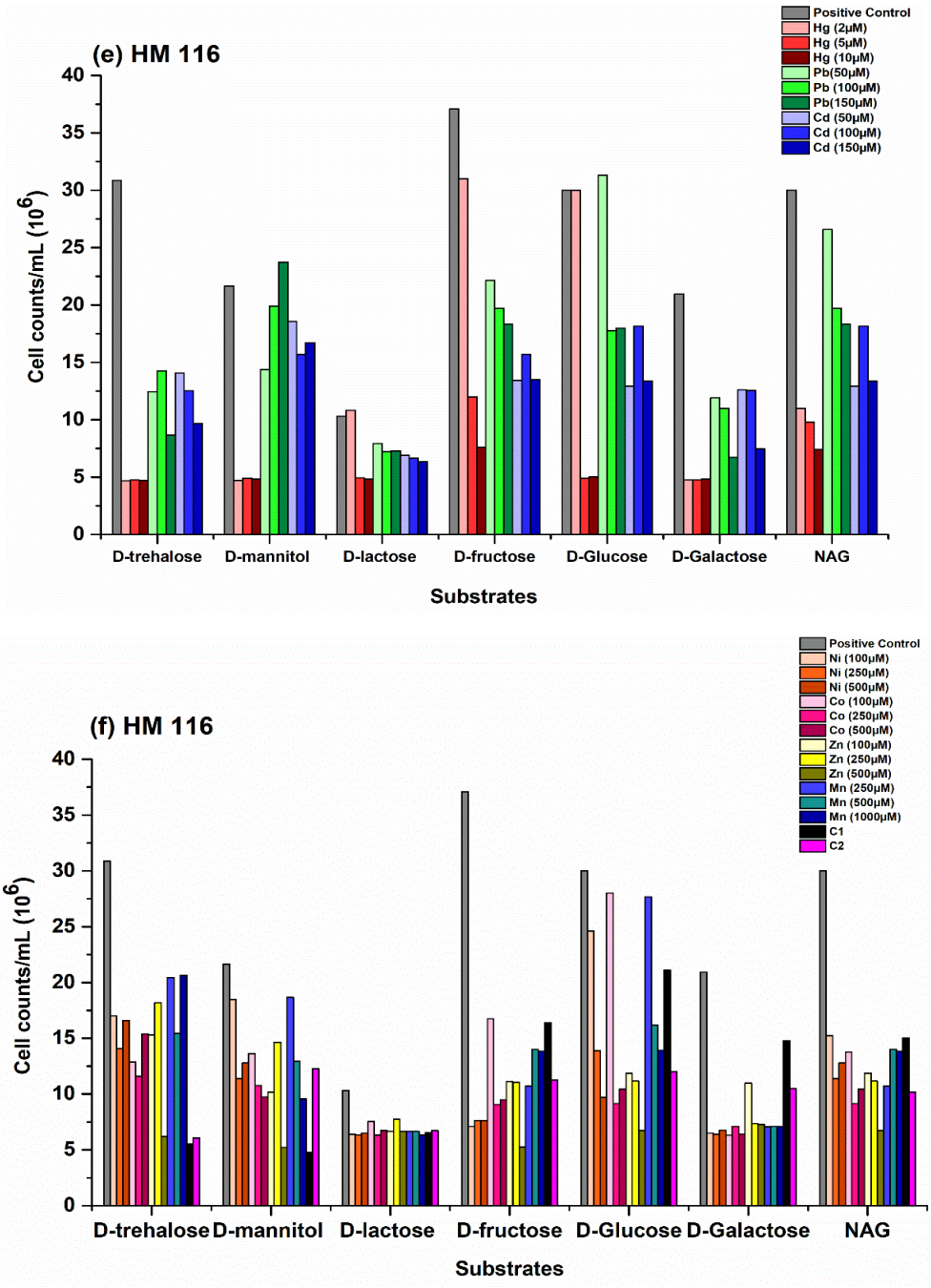


Fig. 5.3. Bacterial growth (expressed in cell counts/ml) in different carbon substrates for the positive control and the different metal amendments for the 14th day of incubation. **Fig. 5.3 (a)** and **(b)** shows the growth response in different carbon substrates for HM11 (*Psychrobacter glaciei*) grown in positive control versus Hg. Pb and Cd and positive control versus Ni, Co, Zn, Mn, C1 and C2. **(c)** and **(d)** shows the growth response of HM12 (*Planococcus halocryophilus*) in various carbon substrates grown with and without metal amendments, **(e)** and **(f)** shows the growth response of HM116 (*Halomonas neptunia*) in various carbon substrates grown with and without metal amendments.

5.3.4. Impact of metals on pigment production

Isolate HM12 (*Planococcus halocryophilus*) was tested for their pigment production in the presence and absence of metals. The pigment produced was orange in colour with its maximum absorbance noted at 450 nm indicating it to be a carotenoid pigment. The pigment extraction was carried out at the 7th-day incubation as well as at the 14th-day incubation of the culture. It was observed that the isolate exhibited the highest pigment production in the positive control. Amendments with different metals and their varying concentrations reduced the pigment production as indicated by the OD values (**Fig. 5.4**). One-way ANOVA with Tukeys test of mean comparisons indicated a significant reduction in the pigment production (expressed as absorbance at 450nm) in all the metal amendments compared to that of the positive control ($p < 0.05$). However, there was no significant difference in the pigment production between the 7th and 14th days of incubation.

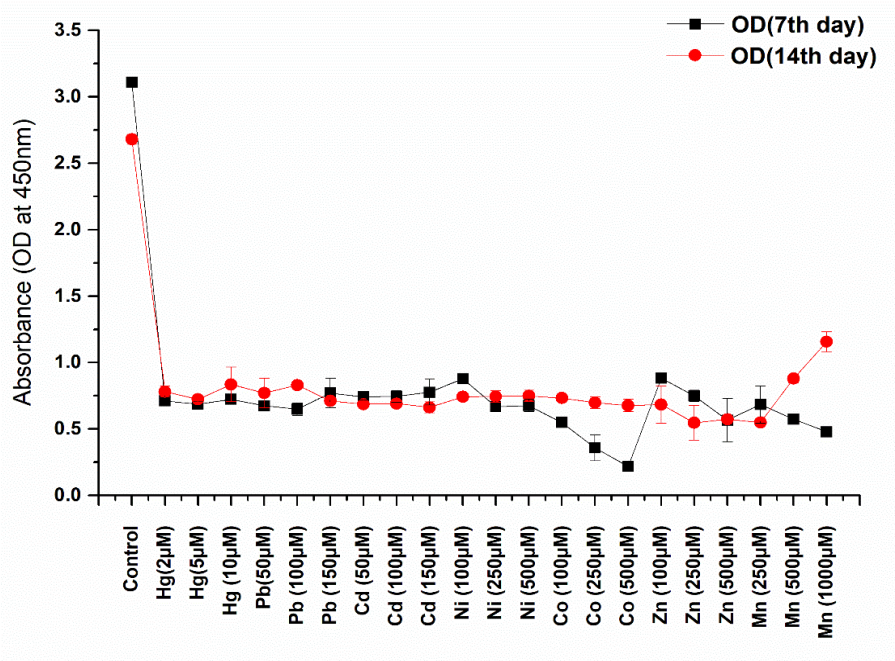
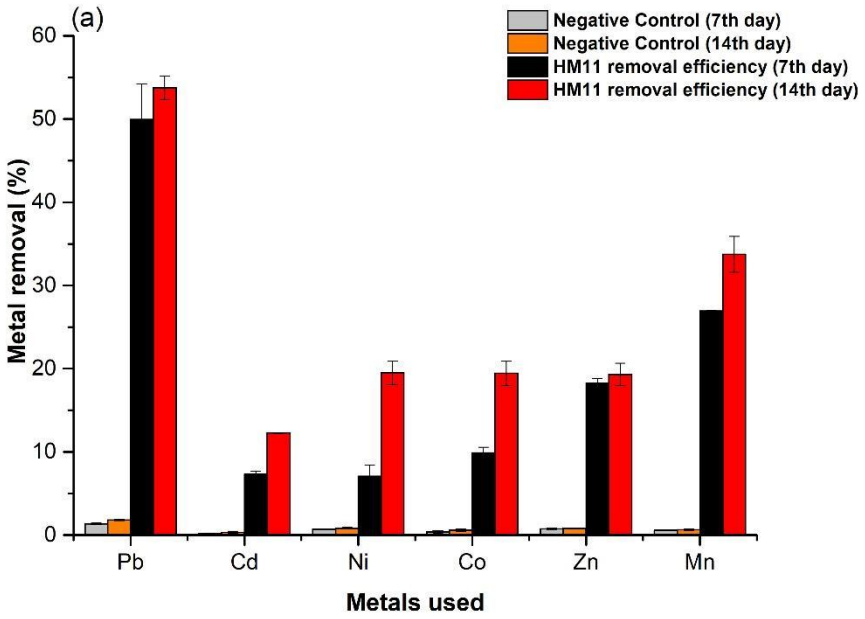


Fig. 5.4. The pigment production of the isolate HM12 (*Planococcus halocryophilus*) as indicated by the absorbance at 450 nm in the control and different metal amendments for 7th (indicated in black) and 14th day (indicated in red) of incubation.

5.3.5. Metal removal efficiency of bacterial isolates

All three bacterial isolates were efficient in removing Pb, Cd, Ni, Co, Zn, and Mn from the broth medium as detailed in **Fig. 5.5**. All the three bacterial isolates HM11, HM12, and HM116 exhibited higher efficiency for the removal of Pb as compared to the other metals. For the bacterial isolate HM11 (*Psychrobacter glaciei*), the metal removal efficiency was in the order Pb > Mn > Ni > Co > Zn > Cd (14^{th} - day incubation). (**Fig. 5.5 (a)**). One-way ANOVA indicated significantly higher metal removal efficiency for the culture HM11 incubated for 14 days in comparison to the culture grown for 7 days, for all metals tested with an exception of Pb and Zn ($p < 0.05$). For the isolate HM12 (*Planococcus halocryophilus*), the metal removal efficiency was in the order Pb > Ni > Mn > Cd > Zn >Co (**Fig. 5.5 (b)**). Similarly, for the isolate HM116 (*Halomonas neptunia*), the metal removal efficiency was in the order Pb > Co > Ni > Mn > Cd > Zn (**Fig. 5.5 (c)**). Significantly higher metal removal efficiency ($p < 0.05$) was noted for the cultures HM12 and HM116 incubated for 14 days in comparison to 7 days.



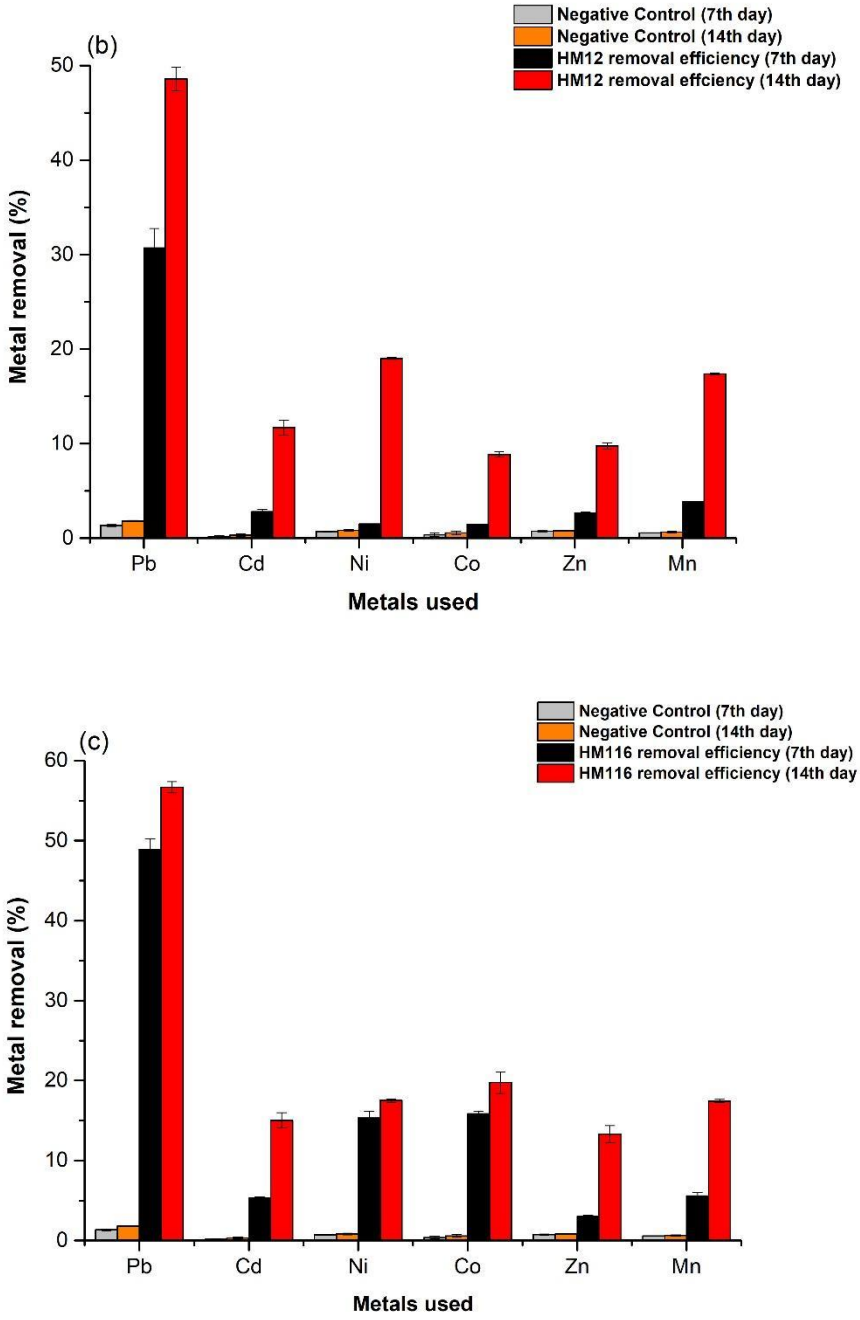


Fig. 5.5. (a) Metal removal efficiency of the bacterial isolate HM11 (*Psychrobacter glaciei*) grown in 20ppm Pb, 10ppm Cd, 25ppm Ni, Co and Zn and 50ppm of Mn compared with that of the negative control (broth enriched with metals but without bacteria), **(b)** Metal removal efficiency of the bacterial isolate HM12 (*Planococcus halocryophilus*) grown in different metals compared with that of the negative control **(c)** Metal removal efficiency of the bacterial isolate HM116 (*Halomonas neptunia*) grown in metals compared with that of the negative control.

It was also observed that at the 0.05 level, there was a significant difference between the metal removal efficiency of all three cultures- HM11, HM12, and HM116. Pairwise comparison of the means for the metal removal efficiency indicated significant differences in the metal removal efficiency between HM11 and HM12 ($p < 0.05$), while no statistical difference was noted between HM11 - HM116 and HM12 - HM116.

To observe the effect of metals on bacterial growth, a growth curve experiment was conducted alongside and results are shown in **Fig. 5.6 (a), (b),** and **(c)**. Bacteria were grown with and without metals and the growth patterns were studied. Statistically, there was no significant difference in bacterial growth noted for different metal amendments in comparison to the control for all the 3 isolates ($p > 0.05$). We could observe significant correlations between the bacterial growth in terms of cell count and the metal removal efficiency for the isolate HM11 (Pearson correlation: $p = 0.04352$, r value = 0.82444). For the isolates HM12 and HM116, there was no correlation noted between the bacterial cell counts and metal removal efficiency of the bacteria.

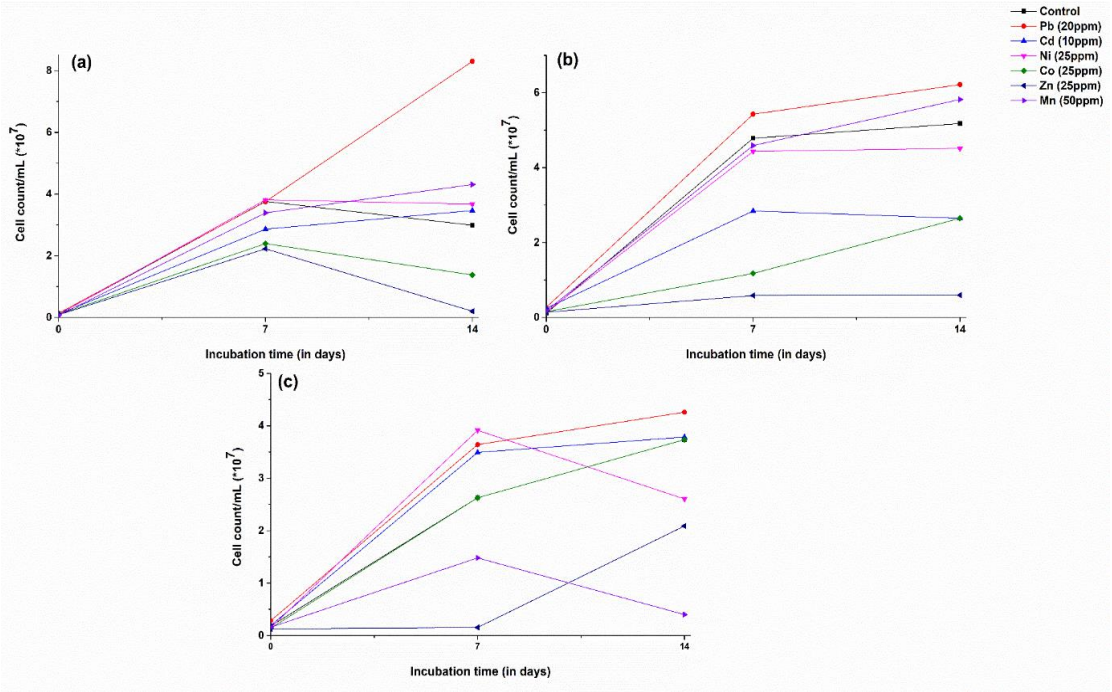


Fig. 5.6. Graph showing the effect of Pb, Cd, Ni, Co, Zn, and Mn versus positive control on growth rate (in terms of cell counts/mL) of **(a)** HM11 (*Psychrobacter glaciei*), **(b)** HM12 (*Planococcus halocryophilus*), and **(c)** HM116 (*Halomonas neptunia*).

5.4. Discussion:

Here, in this chapter, we focused on both the impacts of metals on bacteria and the bacterial role in metal removal. The three isolates selected for our study belong to the genera *Psychrobacter, Planococcus*, and *Halomonas* previously known for their metal bioaccumulation efficiency/ biosorption potential (Abd-Elnaby et al., 2016; Asksonthong et al., 2018; Fernandes et al., 2018; Waghmode et al., 2020) or for the presence of detoxification enzymes (Jung et al., 2018).

From our results, it was observed that the higher concentrations of metals either inhibited or had no effect on the enzyme production properties of the selected bacterial isolates. However, some exceptions like the stimulation of lipase production in *Psychrobacter* sp. (HM11) with Cd, Ni, and Mn amendments were noted. Numerous *Psychrobacter* strains are known for their cold-adapted lipase activity and harbor lipase/esterase genes, particularly encoding cold-adapted lipases/esterases (Yumoto et al., 2003; Kulakova et al., 2004; Zhang et al., 2007; Chen et al., 2011). It has been reported that metal ions and metal ion chelators could affect the lipase activity (Chakraborty and Paulraj, 2009). A study by Novototskaya-Vlasova et al. (2012) reported the stimulatory effect of Mn^{2+} ion on the esterase activity of Polar *Psychrobacter* sp. These 3 metals- Cd, Ni, and Mn also resulted in activating resistance towards the antibiotic Trimethoprim in *Psychrobacter* sp. HM11 which in turn highlights the potential of certain metals to select for antibiotic resistance and enzyme production properties in *Psychrobacter* sp. The inhibitory effect of zinc on enzyme production, antibiotic resistance and carbon substrate utilization profiles of all three bacterial isolates were observed indicating the toxicity of zinc ions on bacterial physiology. Contamination and accumulation of Zn^{2+} ions in the sediment impacting negatively on carbon metabolism and respiratory activities of the bacterial strains was suggested by Nweke et al. (2007).

The impact of metals on antibiotic resistance properties of the three selected bacterial isolates indicated that certain metals are capable of inducing resistance to certain antibiotics while others inhibited bacterial resistance to antibiotics or have no effect on bacterial resistance to antibiotics. The tested metals mostly had a negative impact on

the carbon substrate utilization profiles of the bacterial isolates by causing a significant reduction in bacterial cell counts in comparison to their positive control. Xu et al. (2019) had reported that the increased metal concentrations could repress the efficiency of microbial carbon use with no difference observed between the metal types. Studies have also shown that the different metal enrichments and increased metal concentrations, could cause perturbations in the cellular processes, leading to physiological stress to bacteria (Booth et al., 2015) and in turn, affect the kinetics and patterns of carbon substrate utilization (Konopka et al., 2013).

It was also observed in our study that combination or mixture of metals mostly has an inhibitory effect on bacterial enzyme production, antibiotic resistance, and carbon substrate utilization properties with few exceptions seen, indicating the synergistic effects of metal mix on bacterial physiology and metabolism in comparison to the single metal ions (Kamika and Momba, 2013).

Similarly, there was a significant reduction in pigment production observed for the *Planococcus* sp. HM12 which were enriched with different metals in comparison to the positive control. Furman et al. (1984) suggested that the low levels of toxic metals could inhibit bacterial pigmentation. Another study by Lima e Silva et al. (2012) reported that the presence of metals favored changes in bacterial coloration at low metal concentrations while the same was inhibited at higher concentrations of metals. However, there are no studies till today addressing the impact of metals on Polar bacterial pigmentation even though many of the Polar species favor pigmentation as a survival strategy. Therefore, our study gives a hint at the negative impact of metals on the pigmentation properties of Arctic bacteria.

The presence of metals in the system provides a selection pressure that allows the survival of microbes by evolving mechanisms to resist these metal contaminants (Chaudhary et al., 2017). In this regard, we tried to explore the metal removal efficiency of the three selected bacterial isolates towards different metals. Pb removal efficiency was significantly higher for all three bacterial isolates (53.75% for HM11, 48.57% for HM12, and 56.66% for HM116 cultures incubated for 14 days) in comparison to the metals Cd, Ni, Co, Zn, and Mn ($p < 0.05$). All three bacterial isolates, *Psychrobacter*, *Planococcus*, and *Halomonas* sp. showed the highest removal efficiency for the metal

Pb. The isolate HM11, showed the least removal efficiency for the metal Cd, while for the isolate HM12 and HM116, the least removal efficiency was noted for Co and Zn. Abd-Elnaby et al. (2016) have reported the potential of *Psychrobacter* sp. from Mediterranean seawater and sediment samples to accumulate different metals with the highest efficiency for Pb accumulation while Cadmium was the least accumulated metal ion at a temperature of 30°C incubation. The metal removal efficiency for all three isolates was significantly different for the different metals tested in our study ($p < 0.05$) indicating that the metal removal efficiency by bacterial cells depends on the microbial species and type of metal tested (Kamika and Momba, 2013). Also, no individual isolate in our study showed a high removal rate for all metals tested.

Among the three isolates, the highest removal efficiency for metals was observed for HM11 and HM116 in comparison to HM12. *Psychrobacter* sp. HM11 showed a removal efficiency of 53.75%, 33.76%, and 19.5% for the metals Pb, Mn, and Ni (in the order of their highest removal efficiency noted) (14^{th} - day incubation). *Halomonas* sp. HM116 showed a removal efficiency of 56.7%, 19.75%, and 17.5% for the metals Pb, Co, and Ni while *Planococcus* sp. HM12 exhibited a removal efficiency of 48.6%, 19%, and 17.4% for the metals Pb, Ni, and Mn. Both HM11 and HM116 isolates belong to the phylum Proteobacteria while the isolate HM12 belongs to the phylum Firmicutes. The cell walls of gram-negative bacteria are thinner than gram-positive bacteria (Firmicutes) making the adsorption of heavy metal ions faster through the cell wall. The single thin layer of peptidoglycan in gram-negative bacteria is also favourable for intracellular diffusion of metal ions suggesting their higher metal removal efficiency by accumulation (Fathollahi et al., 2021; Mishra, 2014, Kılıç et al., 2014).

The higher metal removal efficiency for 14^{th} day incubation also coincided with the higher growth rate for each culture (in terms of cell count) at the 14^{th} day incubation. This result was strengthened by the positive correlations noted between bacterial cell counts and metal removal efficiency noted for the isolate HM11, although the same was not evident for the other two isolates.

Thus our results are suggestive that the Arctic metal-tolerant bacterial isolates have the capacity to efficiently remove multiple metals from their system. The intake and subsequent efflux of heavy metal ions by bacteria usually include a redox reaction

involving the metal, suggesting the bacterial role in the biogeochemical cycling of those metal ions. For instance, a higher removal efficiency was noted for the metal Pb which is known to be toxic towards microorganisms and recognized as a causative agent for biological damages in the Arctic (AMAP, 2011). Therefore, our findings strongly suggest that the selected metal tolerant bacterial isolates are suitable candidates exploitable for bioremediation applications and also represent an important ecological afford for monitoring sensitive Arctic ecosystems. Further, exploring the genomic signatures underlying metal resistance for such potential isolates could contribute to a better understanding of the Arctic bacterial resistomes.

Chapter 6.

Genomic signatures underlying metal tolerance in selected Arctic bacterial isolates

Chapter. 6

Genomic signatures underlying metal tolerance in selected Arctic bacterial isolates

6.1. Introduction

Arctic microbiota has been considered both sentinels and amplifiers of global climate change (Vincent, 2010). The key to how Arctic microbes respond to changes in their local environment as well as amplify the global impacts of these changes is to be decoded from their genomes (Edwards et al., 2020). So far, there are many genome-based studies on cold adaptive strategies and metabolic profiles in Arctic bacteria. To highlight a few, the genomic analysis of the bacterial strain *Planococcus halocryophilus*, isolated from high Arctic permafrost, revealed the presence of cold and osmotic-specific adaptive mechanisms, along with increased flexibility of proteins in the organism (Mykytczuk et al., 2013). Analysis of Arctic psychrophilic *Psychrobacter* sp. DAB_AL32B genome content by Ciok and Dziewit, (2019) revealed its overall stress response, and genes conferring protection against various stress factors (i.e., low temperature, increased ultraviolet radiation, oxidative stress, and osmotic pressure). Similarly, genes associated with the degradation of phenolic compounds, nicotine, styrene, ethylbenzene, etc were detected in the Arctic marine *Psychrobacter* strains (Moghadam et al., 2016). However, genome-based studies to understand the resistance mechanisms towards contaminants like metals, in Arctic bacteria are lacking.

It is well-known that bacteria have several molecular mechanisms such as efflux pumps, detoxification, etc. to cope with heavy metal toxicity (Nies, 1999; Lemire et al., 2013). Exposure to metals and other contaminants in the natural environment may promote the spread of resistance elements in microorganisms. For example, metal stress acts as a co-selection agent in the selection of antibiotic resistance genes (ARGs) along with the metal resistance genes (Li et al., 2017). Mechanisms like co-resistance, cross-resistance, and co-regulation mediate the co-selection of elements in bacteria. Co-resistance occurs when genes encoding for antibiotic and metal resistances are close to each other in a genome, particularly when present on plasmids (Pal et al., 2017).

However, cross-resistance mechanism is when the same system, confers resistance to both metals and antibiotics (Baker-Austin et al., 2006). For example, multidrug efflux pumps are wide-spread and well-conserved elements thought to have evolved in microorganisms, known to extrude a wide range of substrates from the cell, not only antibiotics and metals, but also quorum-sensing signals, and bacterial metabolites (Blanco et al., 2016). The third mechanism mediating co-selection occurs through the transcriptional co-regulation of resistance genes. For example, heavy metal ions can increase regulation of efflux systems which results in increased resistance towards antibiotics (Seiler et al., 2012). Studies about understanding such co-selection mechanisms through a bacterial whole-genome-based approach are also lacking in the Arctic.

Therefore, here we tried to decipher the genetic determinants underlying metal tolerance and other co-selective resistance elements among three selected bacterial isolates. These three bacterial isolates were chosen based on metal bioaccumulation potential along with changes in their physiological and metabolic profiles in response to various metal stresses (Detailed in **Chapter 5**). The bacterial isolates chosen include HM11 closely related to *Psychrobacter glaciei,* (99.86%), HM116 closely related to *Halomonas neptunia* (99.57%), and HM12, closely related to *Planococcus halocryophilus* (100%). The study also aims to compare the genomes of these three bacterial species with their phylogenetically related counterparts from both Polar and non-polar environments to decipher how metal resistant gene elements are encoded across closely related strains.

6.2. Methodology

6.2.1. DNA extraction from the selected isolates

All three isolates chosen for the study were isolated from the Kongsfjorden sediments following enrichment in minimal media (1% Nutrient Broth) with different metals (HM11 from Pb amended media; HM12 and HM116 from Mn amended media). The fully grown pure cultures (incubated at 15 °C for 2 weeks) isolated from the respective metal amended media were used for the DNA extraction. In brief, DNA was isolated using the ChargeSwitch gDNA mini bacteria kit (Invitrogen, Carlsbad, CA, USA). The quality of the DNA was assessed by gel electrophoresis (1% agarose gel) and the quantity was estimated by Qubit 4 fluorometer (Thermofisher Scientific, USA) using Qubit 1X dsDNA HS (High Sensitivity) Assay Kit (Thermofisher Scientific, USA). The 16SrRNA gene sequence identity for all 3 isolates was confirmed following the protocol as described in **Chapter 3. Section 3(a).2.5.**

6.2.2. De novo whole genome sequencing and bioinformatics analysis pipeline

The whole-genome sequencing was carried out using Illumina HiSeq 2500 (Illumina, Inc., CA, USA) sequencing platform following paired-end library preparation for 2 × 150bp read length chemistry. 100ng/µL concentration of each sample was used for the whole-genome sequencing. The obtained raw sequence read pairswere quality checked for base quality score distributions, average base content per read, and GC distribution. The adapter sequences, reads containing average quality score of less than 30 were removed from the sequence datasets using AdapterRemovalV2 (Schubert et al., 2016). The good quality read paires were assembled into contiguous sequences (contigs) by the De novo assembly method using the Unicycler assembler (Wick et al., 2017). The assembled genomes were further checked for their completeness and contamination using the bioinformatics tool CheckM v.1.1.3 (Parks et al., 2015). The assembled draft genomes were subjected to predicting the CoDing Sequences (CDS) using Prodigal (Hyatt et al., 2010). The predicted genes were further annotated by matching with the UniProt database using the BLASTX program with an E-value cutoff of 10^{-3}. Circular genome maps for the assembled fasta sequences were generated using the CGView

Server software (https://cgview.ca/) (Grant and Stothard, 2008), and the metal resistant, antibiotic-resistant, and cold stress-related genes were annotated on the circular genome maps using Prokka Annotation. Further, we have also cross-checked with the BacMet2 database for the metal gene annotation (Pal et al., 2014).

6.2.3. Comparative genome analysis

To compare the genome of the selected isolates with the other species belonging to the same genus, the available genomes for each of the 3 genera *Psychrobacter, Planococcus,* and *Halomonas* were used. The NCBI-genome-download 0.2.12 script was used to retrieve all sequenced genomes from the genus *Psychrobacter, Planococcus,* and *Halomonas*. These genomes were further grouped as polar and non-polar species based on their source of isolation/reported geographical location. The quality of downloaded genome assemblies was assessed using CheckM v.1.1.3 (Parks et al., 2015) for quality scores. Only those genomes having greater than 99% completeness and less than 1% redundancy were used for further analysis. The taxonomic lineage of the genomes was also cross-checked by gtdbtk toolkit v.1.5.1 using genome taxonomy reference database version r202 (Chaumeil et al., 2020; Parks et al., 2018). All the genomes which have passed the initial QC were analyzed along with assembled draft genomes from this study using anvi'o v.7.0 (Eren et al., 2015). Pairwise comparison of percent average nucleotide identity (ANI) was calculated using pyANI v.0.2.10 (Pritchard et al., 2016). The contig database was generated for each genome using the "anvi-gen-contigs-database" script. Putative coding DNA sequences (CDS) were predicted for each genome using Prodigal (v.2.6.3). All the genomes were also annotated for predicting metabolic functions using NCBI-COGS, kegg-kofams databases. From the gene annotation results for the genomes, the metal resistant gene elements of the selected isolates in our study were compared to that of the other members of their respective genera. The pangenomic analysis was carried out using anvi'o v.7.0 (Eren et al., 2015), with the following parameters; minbit - 0.5, mcl-inflation 8, and ncbi-blast. Single-copy marker genes (SCGs) were identified across the genomes using Hidden Markov Model (HMM). The identified SCG gene clusters of amino acid sequences were concatenated using the "anvi-get-sequences-for-gene-

clusters" script and aligned using the MUSCLE algorithm (Edgar 2004). The unaligned sequences were removed from the analysis using trimAl (Capella-Gutiérrez et al., 2009). The curated SCG datasets were further subjected to phylogenetic analysis using IQ-TREE (Minh et al., 2020) and ModelFinder Plus (Kalyaanamoorthy et al. 2017) for selection for accurate phylogenetic estimates. The generated tree file was imported into the ANVIO pangenome database for visualization. The phylogenomic tree was generated using the itol software v6 (https://itol.embl.de/) (Letunic and Bork, 2021).

6.3. Results

6.3a. *Psychrobacter* sp. HM11

6.3a.1. Phylogeny and genome assemblies

The 16S rRNA gene sequence of *Psychrobacter* sp. HM11 shared 99.72% similarity with the closest type strain *Psychrobacter glaciei* BLC20019, isolated from the ice core of Austre Lovénbreen glacier in Ny-Ålesund, Svalbard, Arctic (Zeng et al., 2016). The assembly yielded a total length of 3313619 bp with an average GC content of 43.43%. The draft genome of the strain HM11 consisted of 25 contigs, of which 17 contigs have >=1000bp. The largest contig size was 1149726 bp while the smallest contig was 1213bp. The assembled genome showed 99.95% completeness and 0.82% contamination as per the CheckM tool. The functional annotation predicted a total of 2727 protein-coding genes, 3 rRNAs, 44 tRNA genes, and 1 tmRNA (**Table 6.1**). The draft genome sequence of *Psychrobacter* sp. HM11 was deposited in the DDBJ/EMBL/GenBank database under the Accession number **JAKUDE000000000**.

Table. 6.1.

Psychrobacter sp. HM11 genome statistics.

Attribute	Value
Genome size	3.31Mb
GC (%)	43.42%
Total no. of genes	2772
Total RNAs	48
rRNAs	3
tRNAs	44
tmRNAs	1
Protein coding genes (CDS)	2724
No. of contigs	25
Contigs (>= 1000 bp)	17
Length of the largest contig	1149726 bp
N50	779591
N75	262777

6.3a. 2. Genome annotation of HM11 for metal resistance, antibiotic resistance, and stress tolerance genes

Functional annotation of draft genome HM11 revealed the presence of a number of genes encoding resistance to metal ions like mercury (two copies of *merP*, one copy each of *merR*, *merT*, *merC*), arsenic (*arsA, arsB, arsC, arsH, arsM, arsR2*), copper, silver (two copies of *cusR*), cobalt, zinc, cadmium (*czcA, czcB, czcD* (two copies), *zntA, zntR*), and manganese (*mntP*, *mntH*) (**Fig.6.1**).

Antibiotic resistance genes specific to the antibiotics penicillin (*mrcA, mrcB*) along with the presence of genes coding for multi-drug export proteins (*emrB, emrE*), multidrug resistance proteins (*mdtA, mdtB, mdtC, mdtK, mexB*), multidrug/solvent efflux pump membrane transporter protein (*bepF,* 3 copies of *bepA*), beta-lactamase regulation (*ampD, ampC*) were also identified in the HM11 *Psychrobacter* genome. The presence of inner membrane protein gene implicated in the import of specific toxins in gram-negative bacteria (*yciB*), enoyl-ACP reductase (*fabI*) targeting the antibacterial compound, triclosan was also detected. We could also note the presence of stress-related protein genes which included two copies of genes encoding cold-shock protein – *cspA*, one copy of *cspV*, and one copy each of heat shock protein- *hslR* and *hslO* (**Fig. 6.1**).

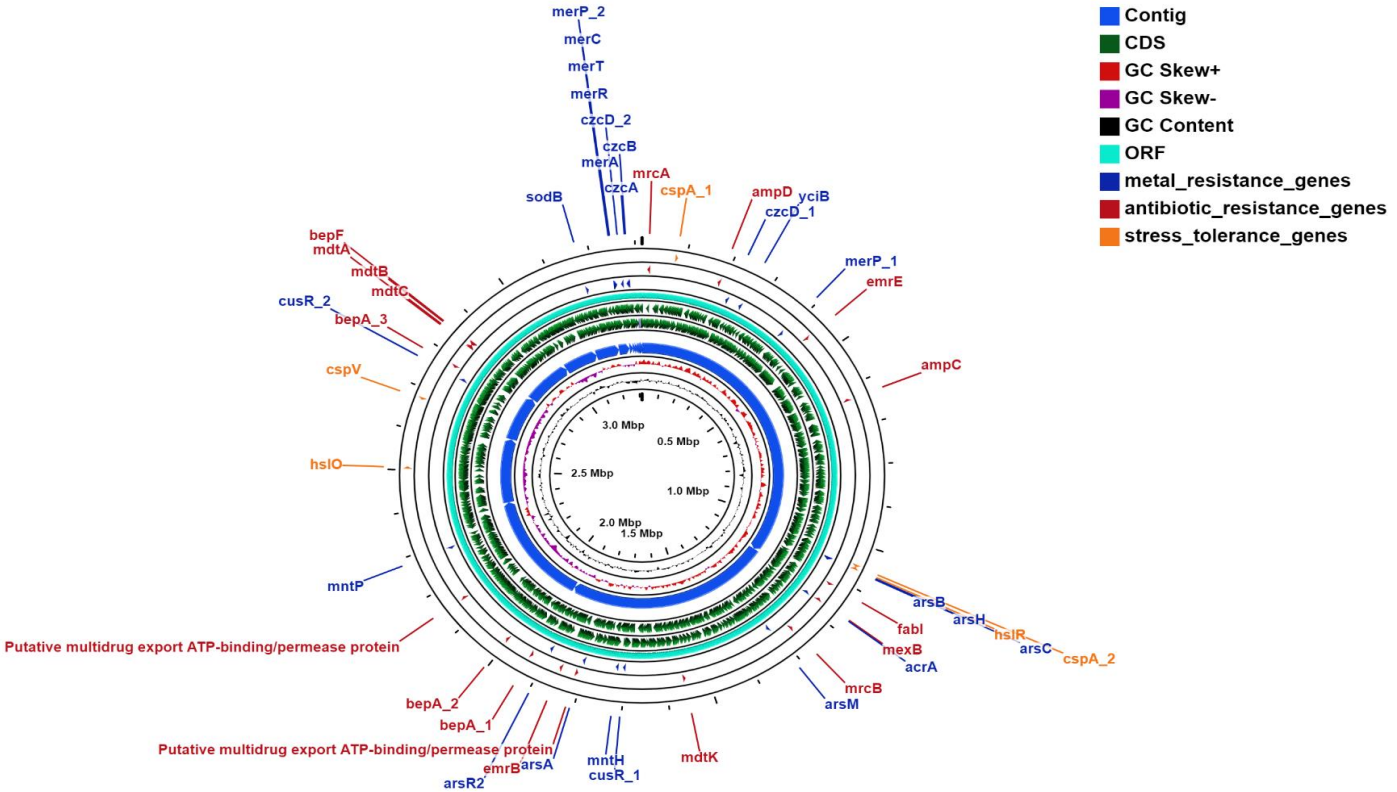


Fig.6.1. Circular representation of *Psychrobacter sp.* HM11 draft genome. The contents of the featured rings (starting with the outermost ring to the center) are as follows. Ring 1: genes involved in stress tolerance, combined forward and reverse strand; Ring 2: antibiotic/multidrug-resistant elements, combined forward and reverse strand; Ring 3: metal resistant gene elements, combined forward and reverse strand; Ring4: Combined ORFs in forward and reverse strands; Ring 5 and 6: CDS in forward and reverse strands; Ring 7: combined contigs; Ring 8: GC skew plot, values above average is depicted in red and below average in purple; Ring 9: plot of GC content; Ring 10: sequence ruler. The figure was produced using CGView ServerBETA (http://cgview.ca/).

6.3a.3. Comparative genome analysis- a pangenome approach

The genome of *Psychrobacter sp.* HM11 was compared with 27 *Psychrobacter* genomes (genomes of the previously characterized valid type strains available in the NCBI database, at the time of study, having greater than 99% completeness and less than 1% contamination). Among the total 28 genomes, 15 genomes including the HM11 genome represented polar species while 13 belonged to non-polar species. Average Nucleotide index (ANI) percentage identity indicated the highest similarity (85.2%) of HM11 to *Psychrobacter okhotskensis* (GCA 904846405.1) with an alignment coverage of 65.7%. The draft genome (HM11) was also found to have minimum of 73 % similarity among the other genomes used in the study. The pangenome analysis revealed a total of 10,408 gene clusters with 73,176 genes across 28 genomes. The phylogenomic tree based on the gene clusters indicated the clustering of the HM11 genome with that of polar species *P. okhotskensis* and a non-polar representative *P. cibarius* **(Fig. 6.2** and **6.3)**. The draft genome (HM11) was found to contain 1016 single-copy core genes (SCGs) and a total of 148 singleton gene clusters. Comparison of metal resistance gene clusters among 15 polar *Psychrobacter* species indicated that about 41% of the metal resistance elements were shared between all 15 species (**Fig. 6.4**). Shared gene clusters included elements resistant to arsenic, zinc, and manganese. Among the metal resistance gene clusters mercuric ion transport protein, mercuric resistance operon regulatory protein, periplasmic mercuric ion binding protein, and mercuric reductase genes were only present in HM11 sp. and *P. nivimaris*. The draft genome (HM 11) exhibited the highest similarity in terms of metal resistance gene elements with that of *P. nivimaris* as indicated in the heatmap (**Fig. 6.4**).

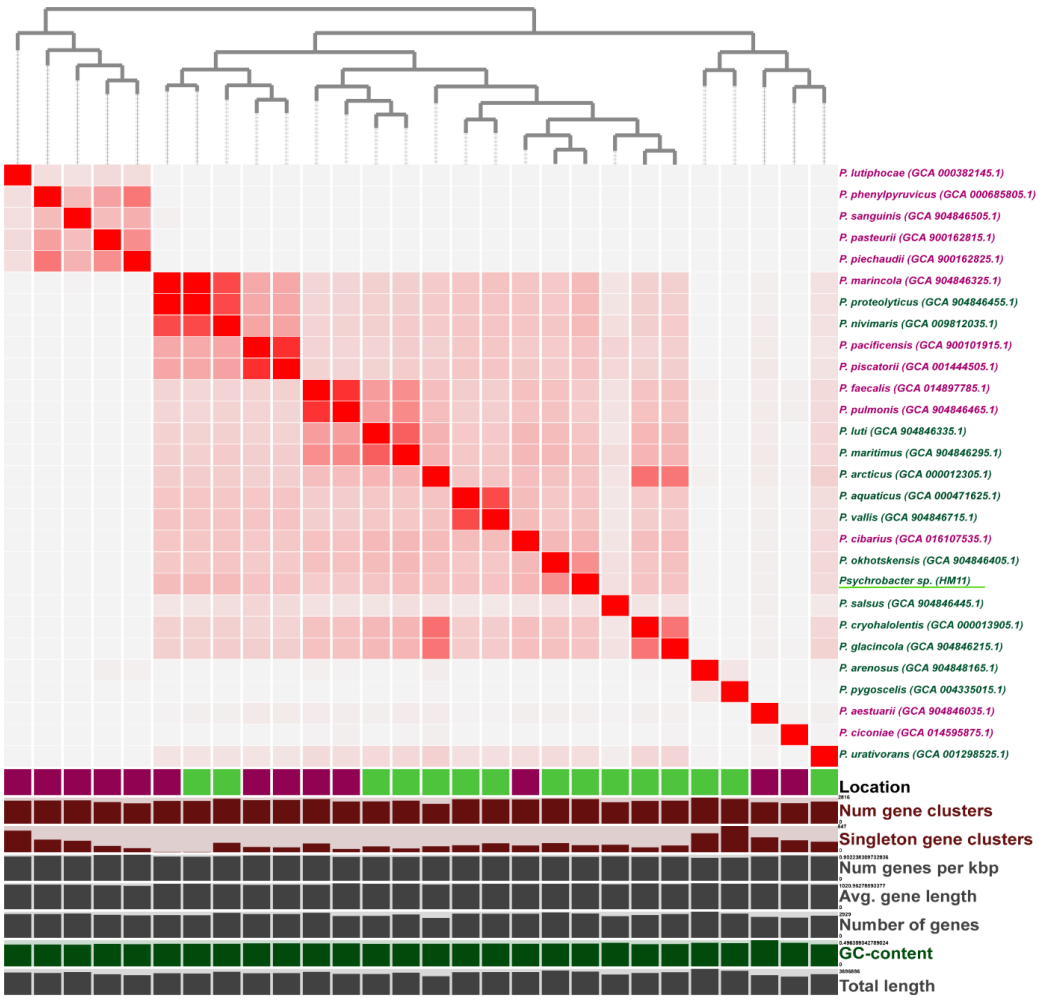


Fig. 6.2. Pangenome analysis describing average nucleotide identities (%) among *Psychrobacter* species. The cladogram on top represents the phylogenomic tree of genomes based on gene clusters. Purple color represents the non-polar species and green color codes for polar species. The total number of gene clusters, number of singleton gene clusters, number of genes, and GC content are also compared between the 28 genomes.

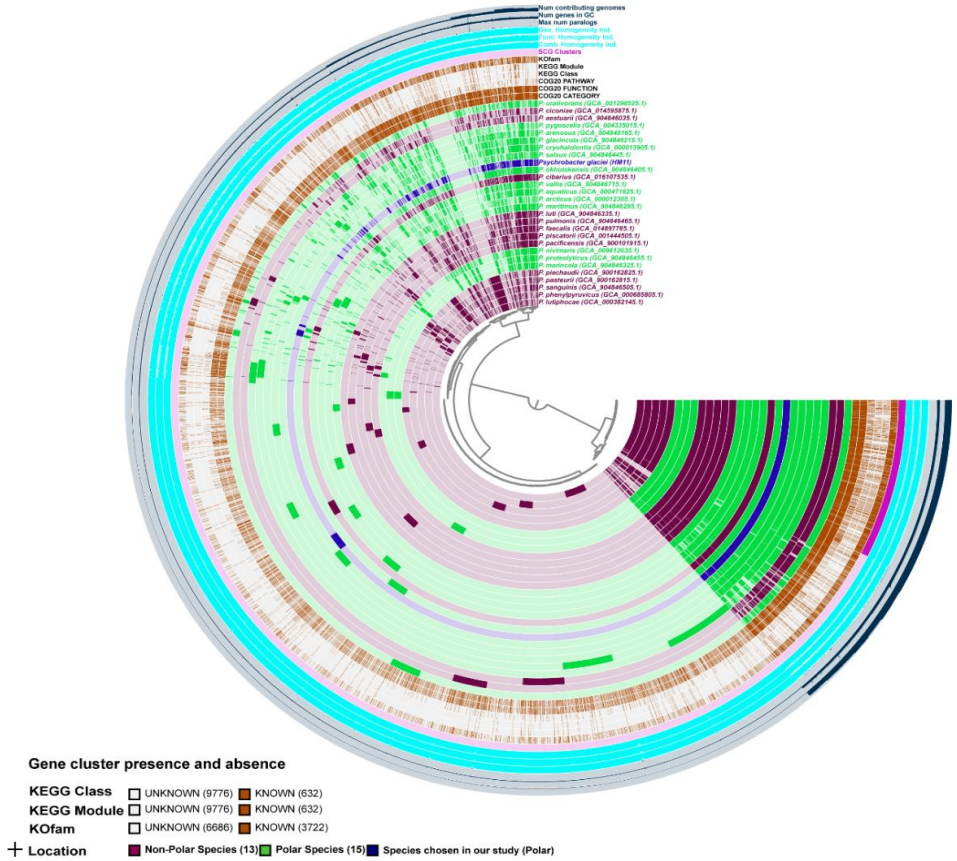


Fig. 6.3. Pangenome analysis revealing the single copy core gene clusters among the *Psychrobacter* species. The analysis revealed 1016 (9.8%) single-copy core gene clusters and 5088 (48.9%) species-specific gene clusters among a total of 10408 gene clusters. Species are grouped into polar and non-polar based on the source of isolation.

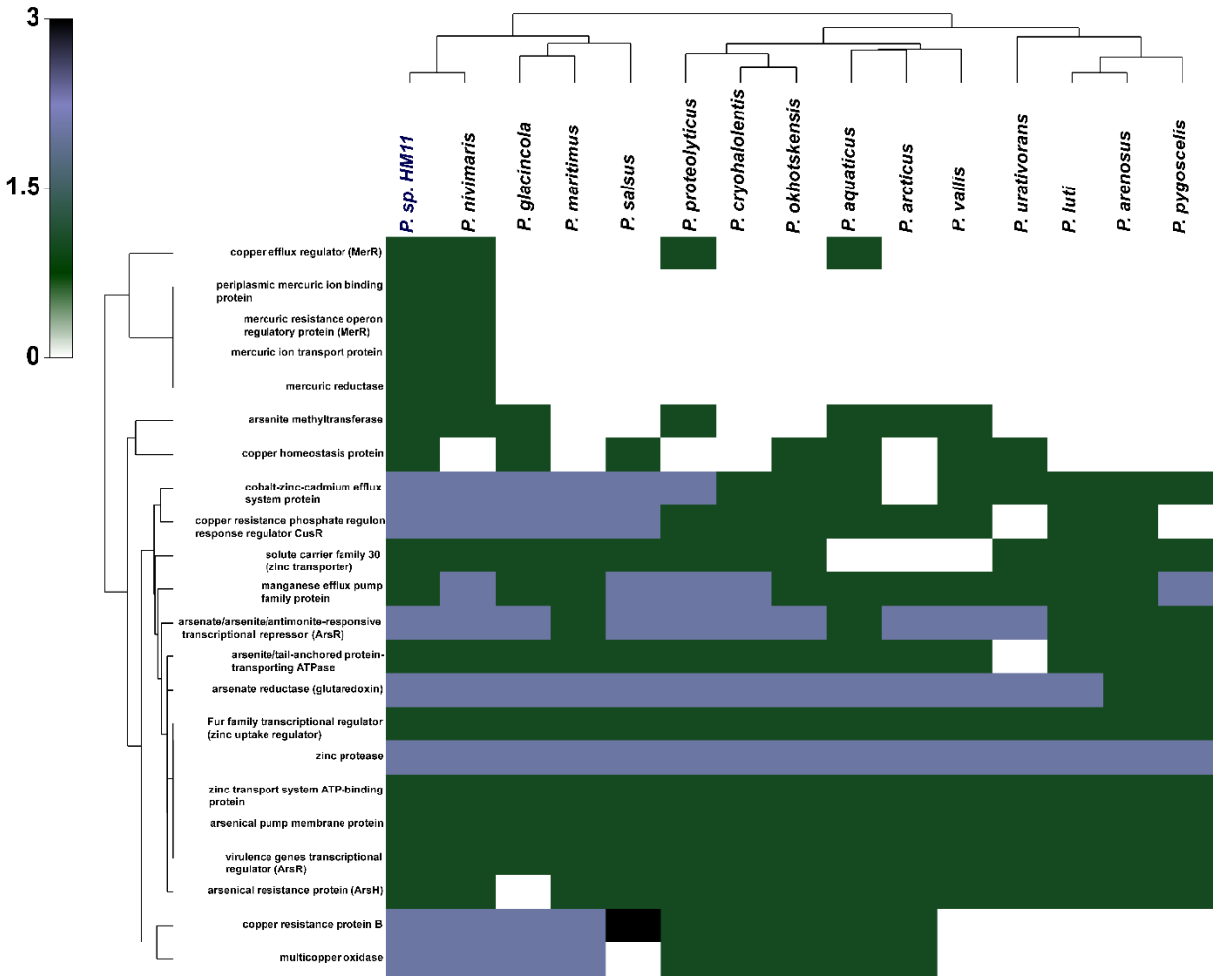


Fig. 6.4. Heatmap depicting the metal gene clusters compared between selected *Psychrobacter* genomes (15 Polar species).

6.3b. *Planococcus* sp. HM12

6.3b.1. Phylogeny and genome assemblies

The 16S rRNA gene sequence of *Planococcus* sp. HM12 shared 100% similarity with the closest type strain *Planococcus halocryophilus* Or1, which was isolated from high Arctic permafrost active layer soil from Eureka, Canada (Mykytczuk et al., 2012). The assembly yielded a total length of 3573775 bp with an average GC content of 39.43%. The draft genome HM12 consisted of 59 contigs, of which 40 contigs have > = 1000bp. The largest contig size was 594551 bp while the smallest contig was 1035bp. The assembled genome showed 99.34% completeness and 0.66% contamination as per the CheckM tool. The functional annotation predicted a total of 3541 protein-coding genes (CDS), 14 rRNAs, 112 tRNA genes, and 2 tmRNA (**Table 6.2**). The draft genome sequence of *Planococcus* sp. HM12 was deposited in the DDBJ/EMBL/GenBank database under the Accession number **JAKVTG000000000**.

Table. 6.2.

Planococcus sp. HM12 genome statistics

Attribute	Value
Genome size	3.328Mb
GC (%)	39.43%
Total no. of genes	3596
Total RNAs	128
rRNAs	14
tRNAs	112
tmRNAs	2
Protein coding genes (CDS)	3541
No. of contigs	59
Contigs (>= 1000 bp)	40
Length of the largest contig	594551bp
N50	231155
N75	98800

6.3b.2. Genome annotation of HM12 for metal resistance, antibiotic resistance, and stress tolerance genes

Genome analysis revealed that the HM12 genome codes for genes encoding resistance to metal ions like mercury (*merA, merR1*), arsenic (*arsA, arsB,* two copies of *arsC*), copper (*copA, copB, copZ*), cobalt, zinc, cadmium (*czcD, znuA, znuB, zitB, zupT, zosA*), manganese (*mntA, mntB*) and chromium (*chrA, chrR*). The presence of superoxide dismutase gene *sodA* was also detected (**Fig.6.5**).

Antibiotic resistance genes specific to the antibiotic tetracycline (4 copies of *tetA*), penicillin (*pbpH, pbpB,* 2 copies of *pbp*) along with the presence of genes coding for multi-drug export proteins (2 copies of *emrY*), multidrug resistance proteins (*mdtC, mdtD,* 2 copies of *mdtH,* 3 copies of *mdtG, bmrA, bmrU*), multidrug/solvent efflux pump membrane transporter protein (3 copies of *bepA*), multidrug ABC transporter permease (*ybhR*) and oxidative stress response protein (*perR*) were also detected in the HM12, *Planococcus* genome. We could also note the presence of stress-related proteins in the bacterial genome (two copies of genes encoding cold-shock protein *cspB,* one copy of *cspC,* two copies of heat shock protein *hslO,* and one copy of *hslV*) (**Fig. 6.5**).

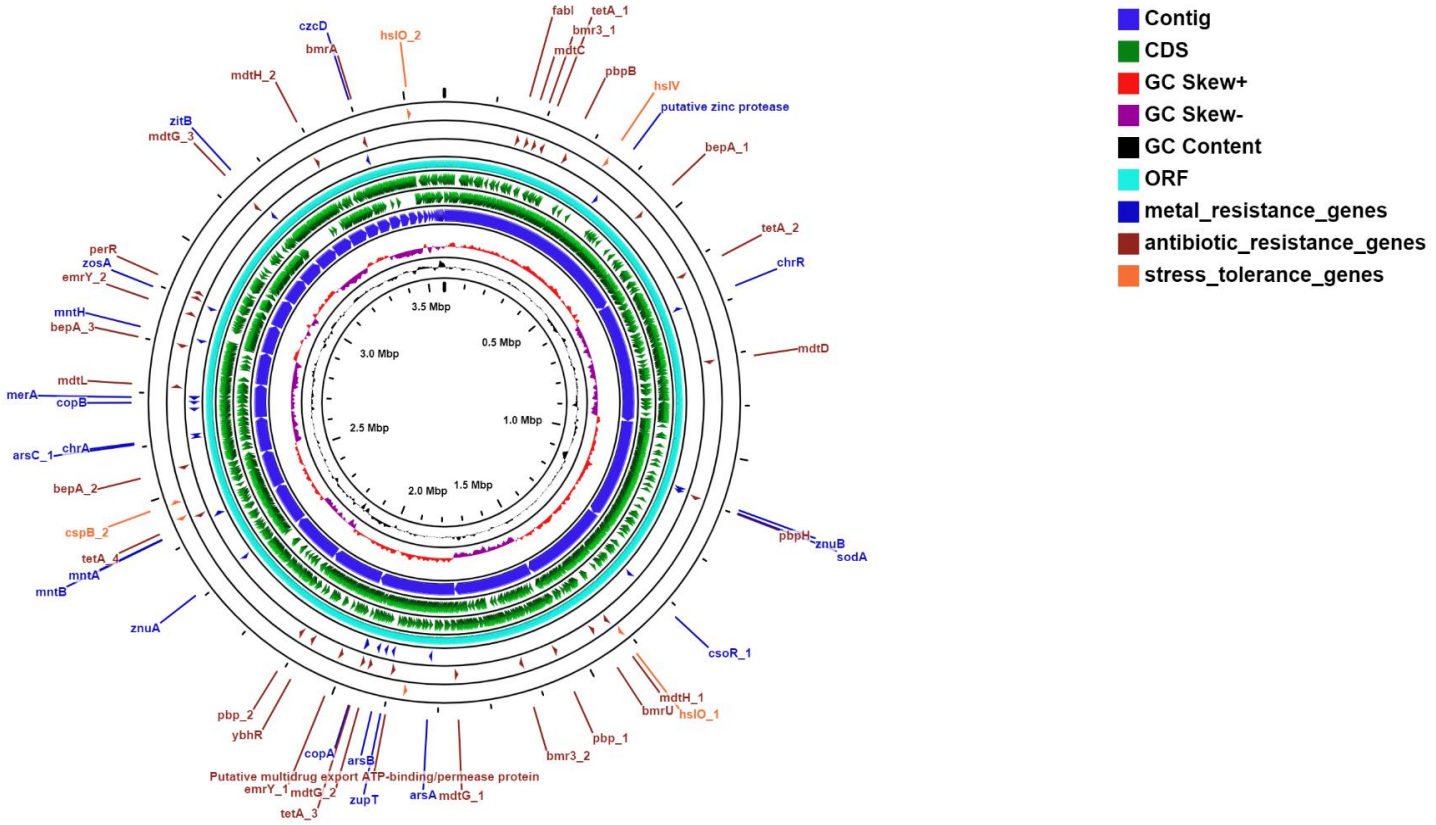


Fig.6.5. Circular representation of *Planococcus* sp. HM12 draft genome. The contents of the featured rings (starting with the outermost ring to the center) are as follows. Ring 1: genes involved in stress tolerance, combined forward and reverse strand; Ring 2: antibiotic/multidrug resistance genes, combined forward and reverse strand; Ring 3: metal resistance gene elements, combined forward and reverse strand; Ring 4: Combined ORFs in forward and reverse strands; Ring 5 and 6: CDS in forward and reverse strands; Ring 7: combined contigs; Ring 8: GC skew plot, values above average is depicted in red and below average in purple; Ring 9: plot of GC content; Ring 10: sequence ruler. The figure was produced using CGView ServerBETA (http://cgview.ca/).

6.3b.3. Comparative genome analysis- a pangenome approach

The draft genome of *Planococcus sp.* HM12 was compared with 24 *Planococcus* genomes available in the NCBI database having greater than 99% completeness and less than 1% contamination. Of the 25 genomes, 10 genomes including our genome of interest (HM12) were represented as polar species while remaining 15 were represented as non-polar species. Based on ANI percentage identity, the draft genome (HM12) were shown to have highest similarity (93.96%) to the genome of *Planococcus halocryophilus* Or1 (GCA 000342445.1) with an alignment coverage of 82.5%. HM12 exhibited >80% ANI percentage identity to that of the species *P. donghaensis* (90.2%), *P.* sp. PAMC 21323 (86.9%), *P. faecalis* (81.83%) and *P.* sp ANT H30 (81.8%) (**Fig.6.6**). The genome HM12 also showed >73 % similarity among all other genomes analysed in the present study. The pangenome analysis revealed a total of 11689 gene clusters comprising 86933 genes among 25 *Planococcus* genomes (**Fig.6.7**). The analysis also estimated the presence of 1135 SCGs along with a total of 215 singleton gene clusters in the draft genome (HM12).

The phylogenomic analysis showed HM12 was closely clustering with that of 4 polar species (*P. halocryophilus* Or1, *P. faecalis*, *P.* sp ANT H30, *P.* sp PAMC 21323) and a non-polar representative *P.donghaensis* **(Fig. 6.6** and **6.7)**.

Comparison of metal resistance gene clusters between the 10 polar *Planococcus* species was also undertaken in the study. Of the total metal resistance gene clusters considered, about 80% were shared between all 10 species (**Fig. 6.8**). Shared gene clusters included elements resistant to arsenic, chromium, cobalt, zinc, cadmium, copper, mercury, nickel, and iron. Superoxide dismutase and metal efflux pump-related genes were also shared between the 10 polar *Planococcus* species. HM 12 sp. exhibited the highest similarity in terms of metal resistance gene elements with that of *P. halocryophilus* Or1 and *P. faecalis* as indicated in the heatmap (**Fig. 6.8**).

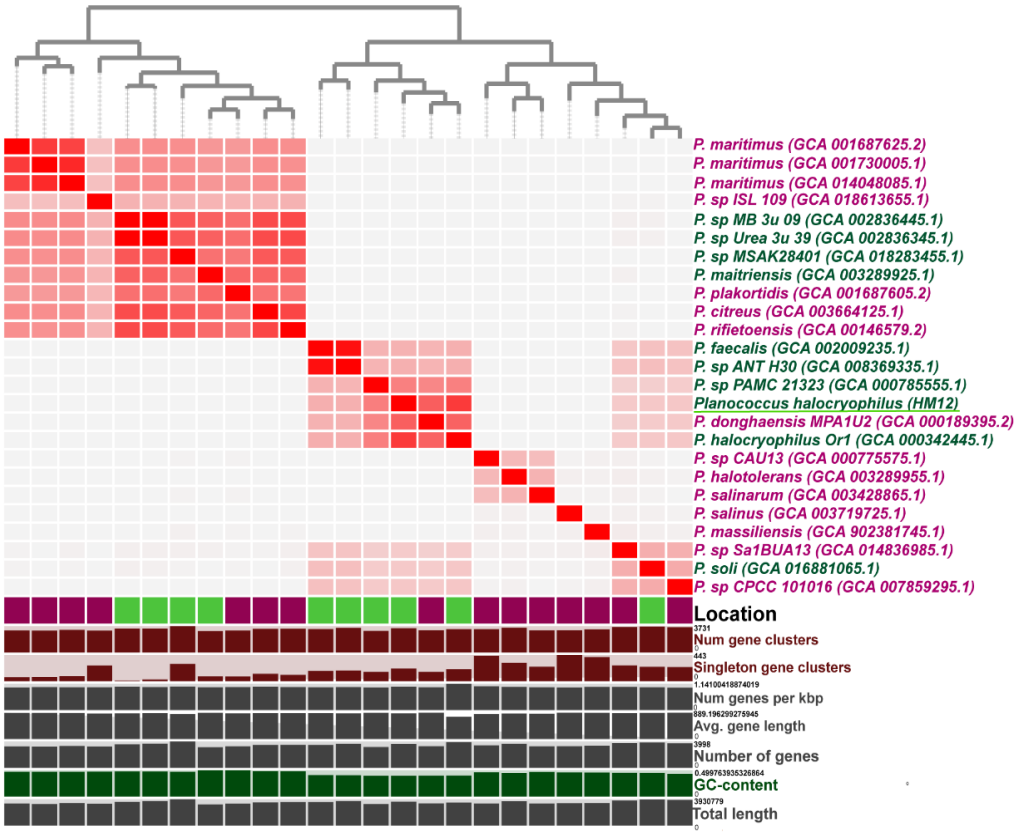


Fig.6.6. Pangenome analysis describing average nucleotide identities (%) among *Planococcus* species. The cladogram on top represents the phylogenomic tree of genomes based on gene clusters. Purple color represents the non-polar species and green color codes for polar species. The total number of gene clusters, number of singleton gene clusters, number of genes, and GC content are also compared between the 25 genomes.

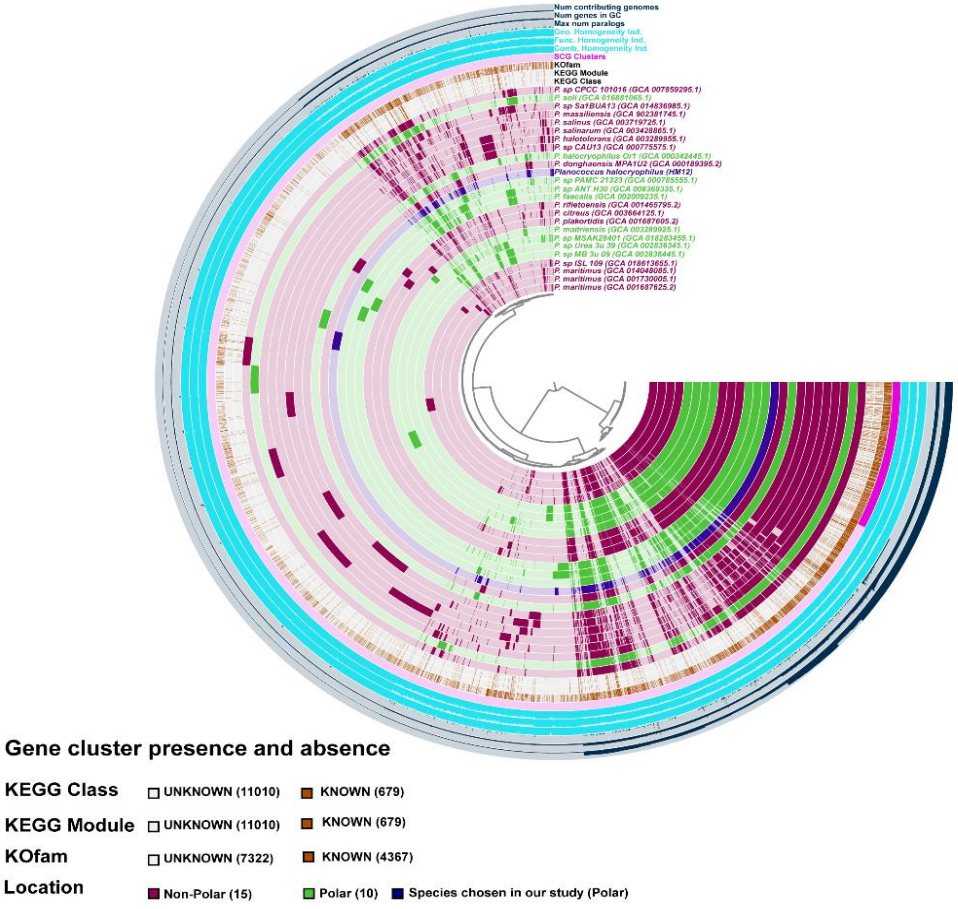


Fig.6.7. Pangenome analysis revealing the single copy core gene clusters among the *Planococcus* species. The analysis revealed 1135 (9.7%) single-copy core gene clusters and 4886 species-specific gene clusters (41.7%) among a total of 11689 gene clusters. Species are grouped into polar and non-polar based on the source of isolation.

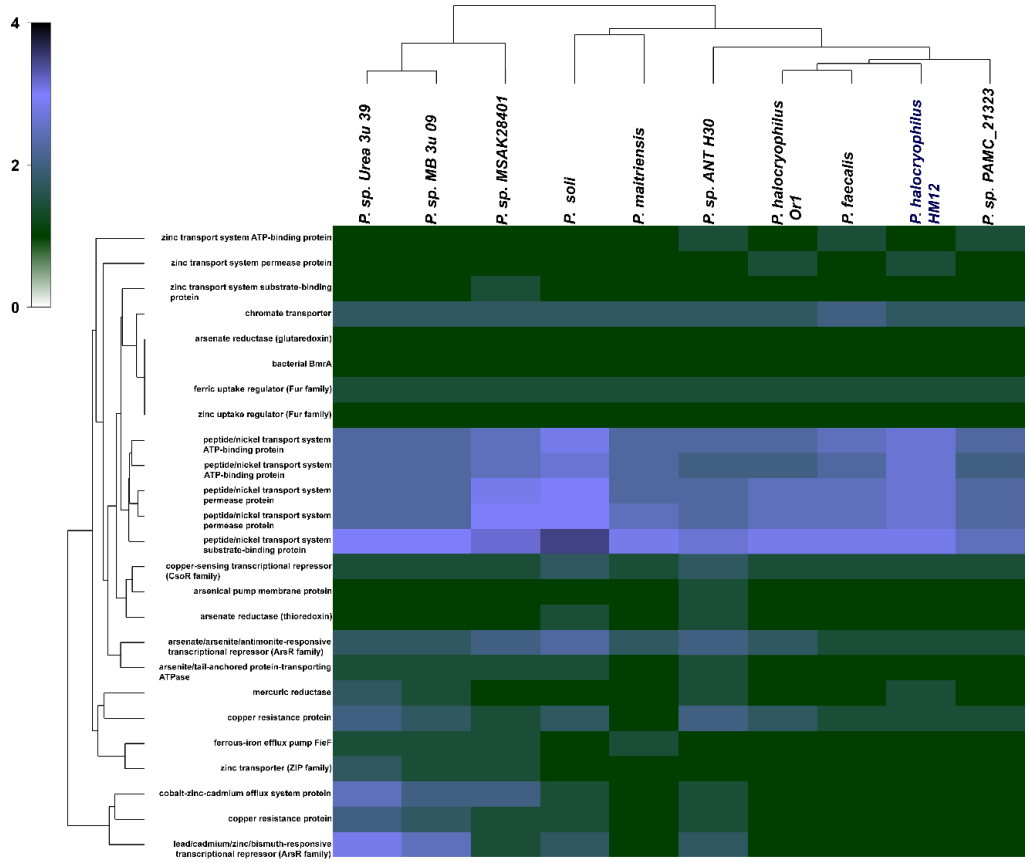


Fig.6.8. Heatmap depicting the metal gene clusters compared between selected *Planococcus* genomes (10 Polar species)**.**

6.3c. *Halomonas* sp. HM116

6.3c.1. Phylogeny and genome assemblies

The 16S rRNA gene sequence of *Halomonas* sp. HM116 shared 99.57% similarity with the closest type strain *Halomonas neptunia*, isolated from deep-sea hydrothermal plume at 2000m depth, from Juan de Fuca Ridge, northeast Pacific Ocean environment (Kaye et al., 2004). The assembly yielded a total length of 4882943 bp with an average GC content of 53%. The draft genome (HM116) consisted of 94 contigs (> = 0bp), of which 60 contigs have > =1000bp. The largest contig size was 923093 bp while the smallest contig was 1102 bp. The assembled genome showed 100% completeness and 1.28% contamination as per the CheckM analysis. The functional annotation predicted a total of 4545 protein-coding genes (CDS), 7 rRNAs, 62 tRNA genes, and 1 tmRNA (**Table 6.3**). The draft genome sequence of *Halomonas* sp. HM116 was deposited in the DDBJ/EMBL/GenBank database under the Accession number **JAKVTW000000000**.

Table. 6.3.

Halomonas sp. HM116 genome statistics.

Attribute	Value
Genome size	4.882Mb
GC (%)	53%
Total no. of genes	4613
Total RNAs	70
rRNAs	7
tRNAs	62
tmRNAs	1
Protein coding genes (CDS)	4545
No. of contigs	94
Contigs (>= 1000 bp)	60
Length of the largest contig	923093bp
N50	188350
N75	123633

6.3b.2. Genome annotation of HM116 for metal resistance, antibiotic resistance, and stress tolerance genes

Genome analysis revealed that the HM116 genome codes for multiple genes encoding resistance to metal ions like mercury (3 copies of *merA*, 4 copies of *merR1*, 2 copies of *merT*), arsenic (*arsC, arsR1*, 2 copies of *arsH*), copper (5 copies of *copA*, 2 copies of *copB*), cobalt, zinc, cadmium (*czcC, czcD, znuC*, 2 copies of *zitB, zur, zupT, zinT*), nickel (*nikR*), silver (*silP*), manganese (*mntA*, 2 copies of *mntB*, 2 copies of *mntP, mntR*), chromium (*chrA1, chrB1, chrR*) and molybdenum (*modA, modB*). The presence of superoxide dismutase gene *sodB, sodC1* and metallothionein gene were also detected (**Fig. 6.9**).

Antibiotic resistance genes specific to broad-spectrum cephalosporins (*ampC*), along with the presence of genes acting as a negative regulator of beta-lactamase expression (*ampD*), transmembrane protein (*ampG*), and transcriptional activator (*ampR*) were found in the HM116 draft genome. Also, genes coding for multidrug resistance proteins (5 copies of *mdtA*, 2 copies of *mdtC, mdtH, mdtN, mexA, mexB*), multidrug/solvent efflux pump membrane transporter protein (4 copies of *bepA*, 2 copies of *bepE*, 2 copies of *bepR, bepF*), putative multidrug export ATP-binding/permease protein and oxidative stress response protein (*perR*) were also detected in the HM116 *Halomonas* genome. Further, the presence of stress-related proteins (2 copies of genes encoding cold-shock protein *cspG*, one copy of *cspV*, one copy each of heat shock protein *hslO, hslR, hslU*, and *hslV*) were also detected in the HM116 genome (**Fig. 6.9**).

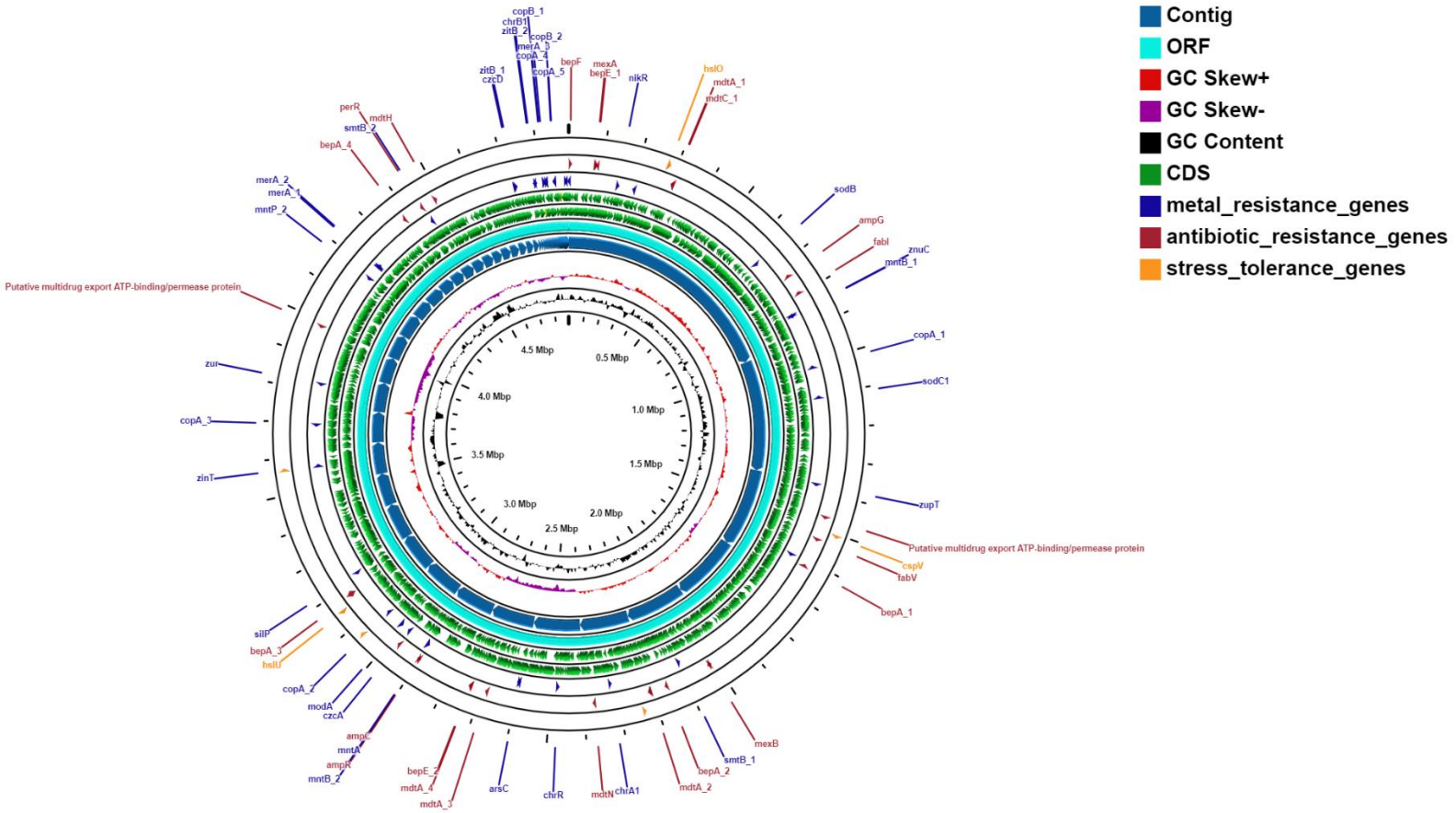


Fig.6.9. Circular representation of *Halomonas* sp. HM116 draft genome. The contents of the featured rings (starting with the outermost ring to the center) are as follows. Ring 1: genes involved in stress tolerance, combined forward and reverse strand; Ring 2: antibiotic/multidrug resistance elements, combined forward and reverse strand; Ring 3: metal resistance gene elements, combined forward and reverse strand; Ring 4 and Ring 5: CDS in forward and reverse strands; Ring 6: combined ORFs in forward and reverse strands; Ring 7: combined contigs; Ring 8: GC skew plot, values above average is depicted in red and below average in purple; Ring 9: plot of GC content; Ring 10: sequence ruler. The figure was produced using CGView ServerBETA (http://cgview.ca/).

6.3c.3. Comparative genome analysis- a pangenome approach

The draft genome of *Halomonas sp.* HM116 was compared with 52 *Halomonas* genomes available in the NCBI database which exhibited greater than 99% completeness and less than 1% contamination. Of the total 53 genomes, 5 genomes including the HM116 represented polar species while 48 belonged to non-polar species. ANI percentage identity indicated the highest similarity of HM116 to the genome *Halomonas maris* (GCA 013371085.1) which was found to be 85%. The next highest ANI similarity was towards *H. titanicae* BH1 (GCA 000336575.1), found to be 83.8%, followed by *H. sedimenti* (GCA 013416325.1) having 83.67% similarity (**Fig. 6.10**). The draft genome (HM116) was also found to have minimum of 72% similarity among the other genomes used in the study. The pangenome analysis revealed a total of 27070 gene clusters with 192279 genes identified across 53 *Halomonas* genomes. The draft genome (HM116) showed the presence of 915 SCGs and a total of 483 singleton genes (**Fig. 6.11**). The phylogenomic tree based on the gene clusters indicated the clustering of the HM116 genome with *H. maris, H. titanicae,* and *H.sedimenti* (**Fig. 6.10**).

Comparison of metal resistance gene clusters between the 5 polar *Halomonas* species was also undertaken in the study. *Halomonas titanicae* species, a non-polar representative was also considered for metal gene comparisons owing to its reported presence from the Kongsfjorden waters and sediments (Sinha et al., 2017; Thomas et al. unpublished). Of the total metal resistance gene clusters considered, 52% were shared between all 6 genomes (**Fig. 6.12**). Shared gene clusters included elements resistant to arsenic, chromium, copper, zinc, cobalt, magnesium, mercury, and nickel. HM 116 sp. exhibited the highest similarity in terms of metal resistance gene elements with that of *Halomonas titanicae* species as indicated in the heatmap (**Fig. 6.12**).

Fig. 6.10. Pangenome analysis describing average nucleotide identities (%) among *Halomonas* species. The cladogram on top represents the phylogenomic tree of genomes based on SCGs. Purple color represents the non-polar species and green color codes for polar species. The total number of gene clusters, number of singleton gene clusters, number of genes, and GC content are also compared between the 25 genomes.

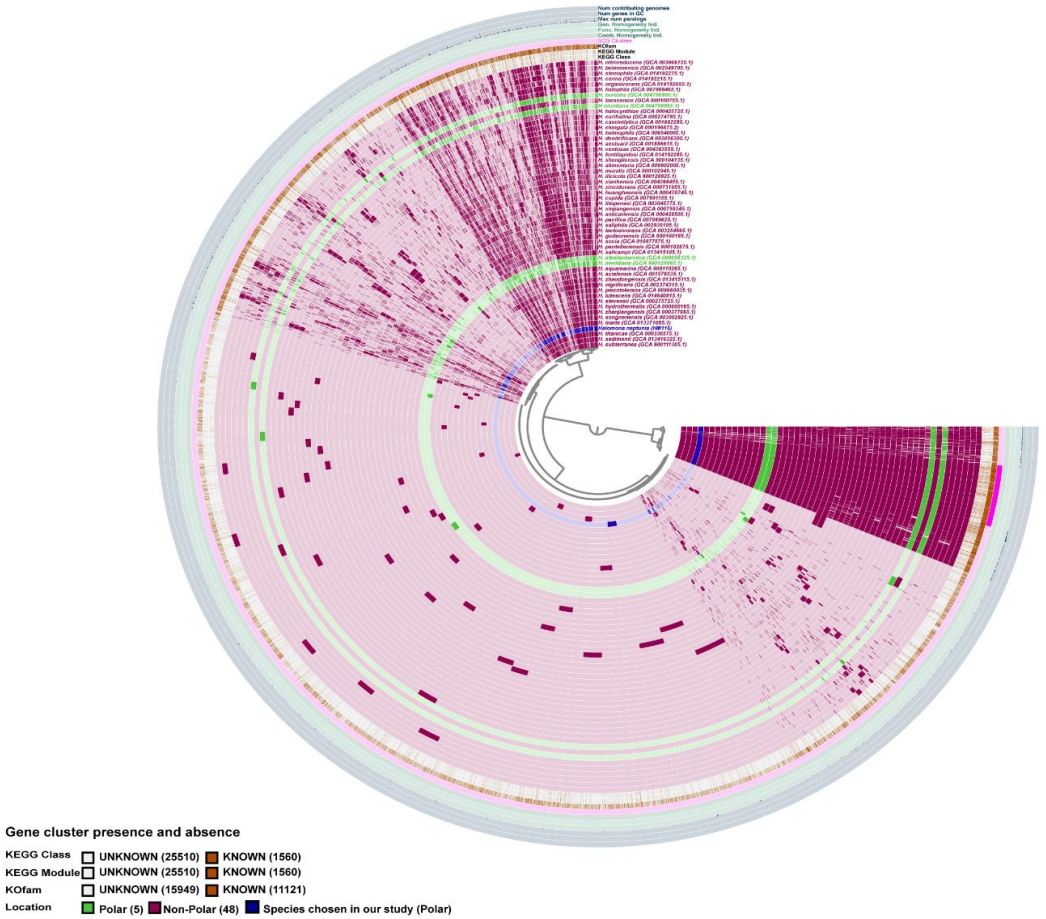


Fig. 6.11. Pangenome analysis revealing the single copy core gene clusters among the *Halomonas* genomes. The analysis showed the presence of 915 (3.38%) single-copy core gene clusters and 13687 species-specific gene clusters (50.56%) among a total of 27020 gene clusters. Species are grouped into polar and non-polar based on the source of isolation.

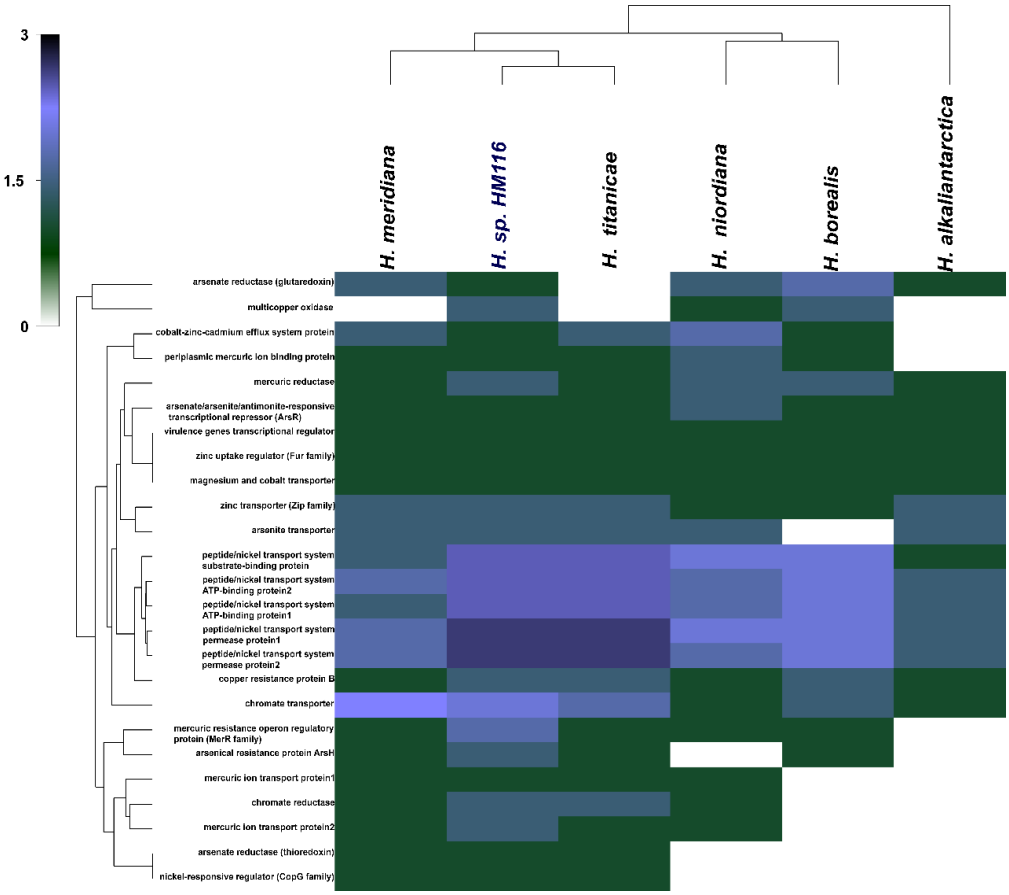


Fig. 6.12. Heatmap depicting the metal gene clusters compared between selected *Halomonas* genomes.

6.4. Discussion

Our study revealed the genetic underpinning of metal resistance, multi-drug/antibiotic resistance, and stress tolerance of the three selected metal tolerant bacterial isolates from the Arctic belonging to *Psychrobacter, Planococcus*, and *Halomonas* genus. The genus *Psychrobacter* includes bacteria that are gram-negative, strictly aerobic, non-motile, cold-adapted, and osmotolerant belonging to the class γ-proteobacteria. They have been frequently isolated from various polar ecosystems including sea-ice, sediments, seawater, and permafrost (Bowman et al., 1997; Romanenko et al., 2002; Vishnivetskaya et al., 2000; Muñoz-Villagrán et al., 2018; Dziewit et al., 2013; Zeng et al., 2015; Ciok and Dziewit, 2019). *Planococcus* species are gram-positive bacteria in the family of *Planococcaceae* (Class Firmicutes). Most *Planococcus* sp. are reported from cold marine environments. They account for 5.8% of the total bacterial community in the Arctic permafrost (Mykytczuk et al., 2012) and can survive in high salinity regions such as Arctic spring channels (Lay et al., 2012). The species *Planococcus halocryophilus* is known to be halotolerant (salinity upto 19%), growing under subzero temperature (-10 °C) and shown to harbor cold-and osmotic stress-tolerant mechanisms as well as cellular mechanisms to thrive in harsh extreme conditions (Mykytczuk et al., 2013). The genus *Halomonas* contains gram-negative rod-shaped, aerobic bacteria belonging to the class γ-proteobacteria. *Halomonas* species have been described as halotolerant, or halophiles, capable of growth in NaCl concentrations of 0.1 to 32.5% (wt/vol) (Vreeland, 2015). Many members of this genera have been isolated from diverse saline Antarctic environments (Poli et al., 2007; James et al., 1990), deep-sea sediments (Wang et al., 2021; Yan et al., 2020; Kaye et al., 2004), and also reported from varying pH and temperature conditions (Kim et al., 2013). These 3 bacterial genera are widespread and evolutionarily successful, while our study revealed presence of a wide variety of resistant elements within their genomes.

Genes encoding homodimeric flavin-dependent disulfide oxidoreductase enzyme mercuric reductase (*merA*), a periplasmic Hg(II) scavenging protein (*merP*), inner membrane-spanning proteins (*merT, merC*) that transport Hg(II) to the cytoplasm for

its reduction by *merA*, and regulatory proteins (*merR*) were present in the mer operon of *Psychrobacter sp*. HM11 genome (Boyd and Barkay, 2012). This indicates the well-developed resistance mechanism in *Psychrobacter* sp. to reduce the local concentration of inorganic Hg (Hg(II)) by reduction to volatile Hg(0), and effectively partitioning Hg(0) to the gaseous phase (Barkay et al., 2003; Lin et al., 2012). The genetic determinants mediating resistance to Co^{2+}, Zn^{2+}, and Cd^{2+} through the formation of a membrane-bound protein complex, catalyzing an energy-dependent efflux of these metal cations was also noted in the HM11 genome. The Czc gene complex present in the genome is composed of the proton-cation antiporter *czcA* which is known to be located in the cytoplasmic membrane, the putative membrane fusion protein *czcB* in the periplasm, and the membrane-bound protein *czcD* located in the downstream regulatory region, required to sense metals through an unknown mechanism (Rensing et al., 1997: Nies, 1992). The Ars operon noted in the HM11 genome involved in the arsenic resistance phenotype consist of genes *arsA, arsB, arsC, arsH, arsM,* and *arsR2*. The gene products of this operon represent a complete arsenic efflux pump in the bacteria where *arsA* forms the primary arsenite transporter which integrates with the other membrane proteins and catalyzes the ATP hydrolysis for the efflux pump (Castillo and Saier, 2010). *arsB* gene encodes for the integral membrane protein enabling extrusion of arsenite from the cell cytoplasm while *arsC* gene codes for arsenate reductase enzymes, able to transform arsenate to arsenite before extrusion of the arsenite. ArsR is a trans-acting transcriptional repressor protein that binds to the promoter region of ars operons, playing a key role in the transcription of the operon (Ben Fekih et al., 2018). Additionally, *arsM* and *arsH* genes code for detoxification systems for organoarsenicals (Yang and Rosen, 2016). Genes coding for manganese uptake (*mntH*) and manganese export from the cell (*mntP*) were also found in the HM11 genome. The presence of these *mnt* genes are indicative of their role in manganese homeostasis within the cell. Genes coding for superoxide dismutase (*sodB*) involved in the destruction of toxic superoxide anion radicals and the transcriptional regulatory proteins functioning in response to increasing levels of copper and silver (*cusR*) and were also noted in the *Psychrobacter* HM11 draft genome.

Multidrug efflux pump genetic elements such as *mdtA, mdtB, mdtC,* and *mdtK* belonging to the RND family (resistance-nodulation-division), *emrE* belonging to SMR superfamily (small multi-drug resistance) along with penicillin-binding protein genes, and beta-lactamase regulator protein genes were also present in the genome. Thereby, suggesting the strong antibiotic resistance mechanisms active in the bacteria despite its polar origin. The GC content of the HM11 genome was well within the range for the genus *Psychrobacter* (38.4-49.6%). The comparative ANI analysis of *Psychrobacter* sp. HM11 with 27 available *Psychrobacter* genomes showed less similarity than the species boundary cut off values (of >96.5%) described by Varghese et al. (2015), suggesting HM11 may belong to novel species.

Interestingly, there were differences in the abundance and distribution of metal resistant gene elements between the Polar *Psychrobacter* species (**Fig. 6.4**). The cluster analysis based on the metal resistant genes revealed close clustering of *Psychrobacter* sp. HM11 with *P. nivimaris*, wherein they shared 21 resistant genes among a total of 22 genes understudy, with an exception of the copper homeostasis protein gene. The presence of mercuric ion related genetic elements such as the periplasmic mercuric ion binding protein, mercuric resistance operon regulatory protein, mercuric ion transport protein, and mercuric reductase gene were observed only in HM11 and *P. nivimaris* while these elements were absent among other polar *Psychrobacter* species. This could be well correlated with the source of isolation of HM11 bacteria *i.e.* the Kongsfjorden outer fjord sediments which were reported to have higher Hg concentrations (Thomas et al., 2021). Genes encoding arsenical pump membrane protein, transcriptional regulator (*arsR*), zinc uptake regulator, zinc protease, and zinc transport system-ATP binding protein were equally abundant in all 15 polar species indicating similar mechanisms for zinc and arsenic uptake and efflux in the polar members.

In the genome of *Planococcus* sp. HM12, genes encoding the enzyme mercuric reductase (*merA*) involved in the reduction of Hg^{2+} (aq) to volatile, less reactive elemental mercury (Hg^0 (g)) and the transcriptional regulatory protein (*merR*) facilitating regulation of mer-operon expression (Møller et al., 2014) were noted. The HM12 genome also had transmembrane protein gene czcD involved in the regulation of cadmium, cobalt, and zinc efflux system, cop operon genes *copA* and *copB* encoding

ATPases for influx and efflux of copper, *copZ* gene encoding a copper chaperone (Reyes et al., 2006), arsenite efflux pump genes *arsA* and *arsB* and two copies of arsenate reductase enzyme coding gene *arsC* (Ben Fekih et al., 2018). The presence of genes involved in the manganese uptake (*mntA*, *mntB*, and *mntH*), its regulation (transcription factor *mntR*) (Zeinert et al., 2018), zinc uptake, transport (*znuA*, *znuB*, *zupT*), and regulation (transcription factor *zur*) (Patzer and Hantke, 1998; Mikhaylina et al., 2018) were also observed. The metal responsive transcriptional regulator genes present in the genome regulate the cellular uptake, efflux, and intracellular trafficking of metal ions (Mikhaylina et al., 2018). The presence of arsenate reductase and mercuric ion reductase genes involved in the detoxification of heavy metal ions were previously reported in the genome sequence of *Planococcus* sp. PAMC21323 isolated from Antarctica (Jung et al., 2018).

The presence of numerous multi-drug efflux pump genes, transporter genes, and genes encoding resistance to antibiotics like tetracycline and penicillin in the *Planococcus* sp. HM12 genome was noteworthy. It has been reported that the drug efflux pumps have multi-faceted implications such as response to a range of stress signals, cell to cell communications, biofilm formation, virulence, etc. (Piddock, 2006), suggesting their role in the overall fitness of bacteria. The presence of multiple cold shock protein (*csp*) genes noted in HM12 sp. which are crucial for their cold-adaptive strategies as indicated by Mykytczuk et al. (2013).

The GC content of the HM12 genome was well within the range for the genus *Planococcus* (39.29-50%). When comparing the abundance of metal resistant gene elements (25 genes considered) between the 10 polar *Planococcus* species, it was noted that all the genes were almost equally abundant among all polar species (**Fig. 6.8**). This in turn gives us a hint at the conserved nature of these metal-resistant genetic elements in polar *Planococcus* species.

The *Halomonas* sp. HM116 genome shown to have a well-evolved Mer operon system having three copies of mercuric reductase gene (*merA)*, two copies of inner membrane-spanning protein (*merT)*, and four copies of transcriptional regulatory proteins (*merR)* (Boyd and Barkay, 2012). The presence of genes encoding - membrane-bound proteins catalyzing energy-dependent efflux of Co^{2+}, Zn^{2+}, and Cd^{2+} (Große et al., 1999),

ATPases for influx and efflux of copper (Reyes et al., 2006), ABC transporter, exporter, and transcriptional regulator involved in manganese and zinc homeostasis (Martin et al., 2015; Waters et al., 2011; Quan et al., 2020), arsenate reductase and organoarsenical oxidase enzymes (Ben Fekih et al., 2018) were also noted in the HM116 genome. Additionally, the molybdate transport system composed of a periplasmic binding protein and an integral membrane protein coded by *modA* and *modB* genes (Grunden and Shanmugam, 1997) and chromium resistance genes (*chrA, chrB*, and *chrR*) also formed part of the HM116 resistome. The genomes of different *Halomonas* strains are known to have metal resistance genetic elements including mercury, cadmium, lead (Saito et al., 2019) arsenic (Lin et al., 2012), zinc (Huo et al., 2014), etc. indicating the innate resistant properties of the species of this genera to detoxify metal pollutants. The presence of different efflux pump mediated multi-drug-resistant genes belonging to RND (resistance-nodulation-division) family transporters (*mdt, mex, bep*) suggests the role of bacterial efflux pump mechanisms in the export antibiotics and other noxious substances across the cell surface (Van Goethem et al., 2018). Beta (β)-lactamase regulator genes (*ampC, ampD, ampG*, and *ampR*) which form the crucial elements conferring resistance against β-lactam antibiotics (like ampicillin, penicillin, etc.) among gram-negative bacteria (O Gutkind et al., 2013) were also noted in the HM116 genome. Similar to the other two genomes *Psychrobacter* sp. HM11 and *Planococcus* sp. HM12, the presence of cold stress-related genes supported their polar signature.

The GC content of the HM116 genome was well within the range for the genus *Halomonas* The comparative ANI analysis of *Halomonas* sp. HM116 with 52 available *Planococcus* genomes showed less similarity than the species boundary cut off values (of >96.5%) described by Varghese et al. (2015), suggesting the novelty of the HM116 genome. When comparing the abundance of metal resistant gene elements (25 genes considered) between the 5 polar *Halomonas* species and the non-polar representative species *H. titanicae*, it was noted that HM116 had higher gene abundance for mer operon regulatory protein gene, mercuric ion transporter protein gene, peptide/nickel transport system ATP-binding protein, peptide/nickel transport system permease protein, peptide/nickel transport system substrate-binding protein in comparison to that

of the other polar species (**Fig. 6.12**). The genome comparison based on the metal resistance gene abundance resulted in the clustering of HM116 with *H. titanicae*, a species previously reported for the presence of gene features like metallopeptidases, metal reductases, uptake regulators, and transporters involved in iron homeostasis (Sánchez-Porro et al., 2013).

The presence of metal resistant genetic elements- *merA, merR, czcD,* and *arsC* involved in the regulation and detoxification of metals Hg, Cd, Zn, Co, and As was noted in all the three genomes. Our genomic analysis indicated the presence of potential efflux system(s) active in all three bacterial genomes underlying the metal and antibiotic resistance. The efflux pump mechanism also forms relevant elements in cell-to-cell communications, virulence, and defense against oxidative stress, thereby influencing the overall bacterial fitness (Alcalde-Rico et al., 2016). Therefore, these efflux pump-related genetic elements must be subjected to future transcriptomics or proteomics investigations. Our study indicated that the three bacterial species HM11, HM12, and HM116 belonging to the genus Psychrobacter, Planococcus, and Halomonas were well-equipped with diverse metal resistant and antibiotic/multi-drug-resistant genetic elements making them ideal candidates for studying the bioremediation potentials of Arctic bacteria. Moreover, psychrophilic/psychrotolerant bacteria can be used to remove contaminants in cold terrestrial sites where mesophilic counterparts do not survive (Jung et al., 2018).

Chapter 7.

Summary and Conclusions

Chapter 7

Summary and Conclusion

The thesis entitled "Bacterial diversity and its interaction with metals in glacio-marine environment of Arctic" deals with a detailed study of the bacterial community structure associated with a land terminating glacier- Vestrebroggerbreen (VB) located in Svalbard Arctic, its foreland channels, and the downstream system of Kongsfjorden. Special emphasis was on the metal tolerant bacterial fraction associated with these connected ecosystems, their response to varying concentrations of metals, and their underlying tolerance mechanisms to withstand the toxic effects of the metal contaminants.

Arctic glaciers and associated fjord systems form distinct ecosystems supporting predominantly microbial life which can withstand the hostile and extreme environmental conditions prevailing in the High Arctic. Microorganisms constitute the basis of trophic networks through their role in nutrient remineralization and associated biogeochemical cycling (Garcia-Lopez et al., 2019; Hoham and Duval, 2001; García-Descalzo et al., 2013). Therefore, it is essential to know how climate change is shaping the distribution and diversity of microbial communities.

The Svalbard Archipelago located in the Arctic Ocean well north of the Arctic Circle between 74° and 81°N is a hotspot to study the climatic impacts in the Arctic since the region has experienced the fastest air temperature increases in recent decades (Nordli et al., 2014). The glacier and marine environments in Svalbard offer numerous distinct habitats such as the glacier snow, glacier ice, meltwaters, terrestrial sediments, tundra soil, fjord waters, fjord sediments, etc. to study the changes in the microbial community structure along with their varying functional profiles. Microbes present in the glacier environments are transported along with the glacier sediments via hydrological networks such as the meltwater channels, streams, and rivers and finally get emptied into the downstream marine systems. The degree to which the downslope

transport of microbes from the glacier systems along the meltwater channels contribute to varying patterns of microbial diversity in the downstream systems of fjords and their ecological dynamics is a lacuna in the Norwegian High Arctic region (Thomas et al., 2020).

Further, in the light of enhanced warming conditions in the Arctic and the reports on the long-range transport of pollutants like metals via atmospheric and oceanic circulation along with the localized inputs (McConnell and Edwards, 2008, AMAP, 2005; AMAP, 2011), it is imperative to understand the trace metal distribution in each of these environmental compartments and how they are impacting the microbiota of the system. The studies on various metal-bacterial interactions, different bacterial mechanisms to tolerate the metal contaminants, and how they contribute to lower/ transform the toxic levels of metals in the Arctic are lacking. Such studies are important since suitable metal tolerant Arctic bacterial isolates can be utilized for bioremediation applications and further represents ideal ecological indicators for monitoring the sensitive Arctic ecosystems.

Hence, the objectives of the study were:

- To understand the bacterial diversity of glacio-marine environment of Arctic through culture-dependent and culture-independent approach
- To assess the diversity of metal tolerant bacteria from the glacio-marine ecosystem of the Arctic
- To understand the metal-bacterial interactions so as to elucidate the role of bacteria in modulating the impact of metals in the glacio-marine environment

For the study, snow, ice, and sediment samples were collected from the VB glacier, meltwater and sediment samples were collected from different points along the foreland channels, and water and sediment samples were collected from the downstream system of Kongsfjorden during the Indian Arctic expedition 2017 (August-September). Both field observations and laboratory-based experiments were carried out for the fulfillment of the objectives. In brief, the bacterial community structure associated with each of these systems was investigated following both the

conventional culture-dependent method as well as the culture-independent high throughput amplicon sequencing approach. On-site measurements of temperature, total dissolved solids, salinity, and dissolved oxygen were carried out at the time of sampling. Measurement of other environmental parameters such as nutrients, major anions, total organic carbon, and trace elements was also carried out systematically to elucidate their role in structuring the bacterial community composition (Thomas et al., 2020). The metabolic potentials of the Arctic sedimentary bacterial communities were investigated in detail using the community-level metabolic profiling and carbon substrate utilization studies of retrieved isolates at varying temperatures, to assess their carbon substrate utilization responses in the terrestrial versus fjord sedimentary environment (Thomas et al., 2021).

The metal tolerant bacterial fraction was assessed by enrichment of the glacier and fjord samples with metals such as Hg, Pb, Cd, Ni, Co, Zn, Mn, and their combinations. Multi-metal tolerant bacterial isolates were selected for the determination of the Minimum Inhibitory Concentration (MIC) for each of the metals and their antibiotic resistance properties were also studied. Further screening of the metal tolerant bacterial isolates based on their MIC values helped to shortlist a few potential candidates for the different metal-bacterial interaction studies. The chosen bacterial isolates were closely related to the species *Psychrobacter* (HM11), *Planococcus* (HM12), and *Halomonas* (HM116). These isolates were studied for understanding the impact of metals (Hg, Pb, Cd, Ni, Co, Zn, Mn, and their combinations) on their enzyme production, antibiotic resistance, carbon substrate utilization, nitrate reduction, and pigment production properties. Similarly, the bioaccumulation potential and metal removal efficiency of the selected bacterial isolates were estimated following an enrichment experiment and measurements of metal concentrations using an ICP-OES (Thermo scientific, iCAP 7000 series, UK). Detailed De-novo whole-genome analysis of the three selected bacterial isolates was also carried out to understand the genomic mechanisms underlying metal resistance, antibiotic resistance, and other stress tolerance.

The salient features of the study are as follows:

- Significant variation (R = 0.873, p = 0.001) in the bacterial community structure between the Vestrebroggerbreen valley glacier, its associated meltwater channels, and the downstream marine system of Kongsfjorden was observed suggesting the influence of environmental variables and dispersal vectors in determining the patterns of bacterial diversity. Similarity percentage (SIMPER) analysis of the Operational Taxonomic Units (OTUs) showed that OTU 1105280 (9.15%) and OTU 331 (6.5%) belonging to *Burkholderiaceae* (β-proteobacteria) and OTU 101660 (5.76%) and OTU 520 (5.07%) belonging to *Rhodobacteraceae* (α-proteobacteria) contributed maximum to the overall dissimilarity between the sampling sites. The dominance of sequences belonging to class β- Proteobacteria was seen in the glacier snow, ice, and meltwaters (MW) while a relatively higher abundance of OTUs belonging to α-Proteobacteria and Verrucomicrobiae demarcated the fjord waters. The VB snow exhibited the lowest diversity with unknown genera belonging to *Burkholderiaceae* dominating the community while a highly diverse (Shannon index between 6.26 and 10.67) bacterial community in the MWs was observed, wherein the dominant bacterial genera were *Flavobacterium, Leptolyngbya, Polaromonas, Methylotenera,* and *Bdellovibrio*. The presence of many unknown taxa in the MWs in both culture-based and high throughput sequencing studies highlights the importance of such transient niches as conducive hotspots for unknown microbiota. The bacterial community from the MWs were found to be true signatures of the glacier ecosystem while the Kongsfjorden bacterial fraction mostly represented heterotrophic marine taxa influenced by warm Atlantic waters and the presence of organic matter. Among the various environmental parameters measured, nutrients (NO_3^- and SiO_4^{2-}) were found to exhibit a strong association with the MW bacterial community while temperature, trace metals, Cl^- and SO_4^{2-} ions were found to influence the fjord bacterial community (Thomas et al., 2020).

- Phylum Proteobacteria and Bacteroidetes dominated both terrestrial and fjord sediments. Phylum Verrucomicrobia and Cyanobacteria were abundant in terrestrial sediments while Epsilonbacteraeota and Fusobacteriia dominated the fjord sediments. Significant differences were observed in the carbon substrate utilization profiles between the terrestrial and fjord sediments at both 4 °C and 20 °C incubations ($p < 0.005$) implying the adaptive responses of bacterial members to utilize the available substrates in the varying natural environment. Utilization of N-acetyl-D-glucosamine, D-mannitol, and Tween-80 by the sediment communities and bacterial isolates from both systems, irrespective of their temperature incubations suggested the affinity of bacteria for such substrates as energy sources and their survival strategies in cold environments. The significant influence of temperature on the growth response of terrestrial and fjord isolates towards different carbon substrates was indicated in our study, with phylogeny found to be a less important structuring component of bacterial metabolic potential. The dominance of class γ-proteobacteria observed in the fjord sediments along with the preferential utilization of carbohydrates and complex polymers in the metabolic profile of bulk sediments indicated their potential role in complex organic matter degradation in the fjord. Higher taxonomic diversity with the presence of diverse rare taxa was noted in the terrestrial sediment VB along with their higher affinity to utilize amino acids, amines, and amides (Thomas et al., 2021).
- Higher trace metal concentrations were noted in the sediments as compared to the water samples from terrestrial and fjord systems. The highest values of Hg (90.9 µg/Kg), Pb (7.9 mg/Kg), Cd (0.2 mg/Kg) were observed in the outer fjord station KNS1 sediment. Similarly, the highest concentration of Ni (40.8 mg/Kg) was noted in the VB snout sediment while the highest concentration of Co (13.2 mg/Kg), Zn (223.9 mg/Kg), and Mn (362 mg/Kg) were noted in the glacier foreland sediment BR. The higher concentrations of elements like Ni, Zn and Mn observed in the terrestrial sediments suggest the possible terrestrial source of these elements into the fjord waters. Also, it can be linked to the abundance of soluble rocks such as carbonates and sulfides in the glacierized areas and the possible enrichment of these elements via the contact of water with glacial debris (Dragon and Marciniak, 2010).

It was also observed there was a significant difference between the water and sediment samples in terms of metal concentrations irrespective of the sampling locations ($p < 0.05$).

- The decreasing order of toxicity of metals based on the viable counts from each of the amendments for the different glacio-marine samples was in the order Hg > Zn > Co > Cd > Pb > Mn > Ni. It was also evident that the Kongsfjorden samples yielded the highest total retrievable bacterial counts in different metal amendments as compared to the glacier snout and foreland samples, suggesting the presence of more resistant bacterial members in the fjord as compared to the glacier samples. Irrespective of the higher metal concentrations observed for the sediment samples, there was no significant variation between the terrestrial sediment and terrestrial waters and fjord sediment and fjord waters in terms of viable count. An only significant difference ($p < 0.05$) in the viable counts was noted between the terrestrial and fjord locations suggesting the stronger influence of sampling location (terrestrial and fjord) on the total retrievable bacterial counts for different metal amendments.
- The dominant metal tolerant bacterial fraction belonged to the class γ-proteobacteria (45% of the total), α-proteobacteria (19.8%), and Actinobacteria (15.8%). Species belonging to the genus *Pseudomonas* were noted from all the sampling locations and all the metal amendments suggesting their inherent metal resistance properties. The presence of *merA*, *czcA*, *czcC,* and *czcD* genes in the metal tolerant bacterial fraction from the terrestrial and fjord samples indicated that metal resistant genes (MRGs) are prevalent even in environments with limited anthropogenic influence. Concerning *merA* and *czcA* genes, it was noted that the gene sequences differed for different bacterial species while for *czcC* and *czcD* genes, conserved gene sequences were present in phylogenetically different species.
- Based on the multi-metal tolerance potential, 20 bacterial isolates were short-listed from the study, which belonged to the genera *Psychrobacter, Halomonas, Planococcus, Pseudomonas, Brucella, Hoeflea, Devosia, Salinibacterium, Staphylococcus, Rhodococcus*, and *Dietzia*. From their MIC testing, it was

observed that the highest concentrations of metals which allowed the bacterial growth were Hg - 10µM, Pb, and Cd - 250µM, Ni, Co, and Zn - 1mM and Mn - 10mM. This indicated the highest toxicity of Hg as compared to the other metals tested in the study. The study also showed that the glacier and the fjord system have different metal tolerant bacterial species exhibiting varying levels of tolerance towards the metals tested. Also, we could observe that the resistance phenotypes in the study were not being influenced by their phylogenetic relatedness. The multiple antibiotic resistance noted for metal tolerant isolates, especially towards the β-lactams and penicillin, implied the co-occurrence of genes for antibiotics and metal resistance within their genomes.

- The further screening of the metal tolerant bacterial fraction lead to the identification of a three potential bacterial species - HM11 closely related to *Psychrobacter glaciei,* (99.72%), HM116 closely related to *Halomonas neptunia* (99.57%), and HM12, closely related to *Planococcus halocryophilus* (100%), exhibiting the highest MIC levels towards different metals tested. It was observed that the higher concentrations of all metals either inhibited or had no effect on the enzyme production properties of the selected bacterial isolates. Exception was noted for *Psychrobacter* sp. (HM11) which had shown stimulation of lipase production with Cd, Ni, and Mn amendments in comparison to the positive control. Metals impacted the antibiotic resistance properties of the selected bacteria by either inducing resistance to antibiotics, inhibiting bacterial resistance making bacteria susceptible to antibiotics or not affecting the bacterial resistance to antibiotics. The tested metals mostly had a negative impact on the carbon substrate utilization profiles of the bacterial isolates by causing a significant reduction in bacterial cell counts in the different carbon substrates. There was also a significant reduction in the pigment production for the *Planococcus* sp. HM12 which was enriched with different metals in comparison to their positive control grown without any metals. The combination of metals mostly had an inhibitory effect on bacterial enzyme production, antibiotic resistance, and carbon substrate utilization properties indicating the synergistic effects of metal mix on bacterial physiology and metabolism in comparison to the single metal ions.

- The selected bacterial isolates could efficiently accumulate the different metals tested in our study. The metal removal efficiency for all three isolates was significantly different for the different metals tested ($p < 0.05$) indicating that the metal removal by bacterial cells depends on the microbial species and type of metals tested. The highest removal efficiency for metals was observed for HM11 (*Psychrobacter* sp.) and HM116 (*Halomonas* sp.) in comparison to HM12 (*Planococcus* sp.). Pb removal efficiency was significantly higher for all 3 bacterial isolates (53.75% for HM11, 48.57% for HM12, and 56.66% for HM116 cultures incubated for 14 days) in comparison to the metals Cd, Ni, Co, Zn, and Mn ($p < 0.05$).
- The genomic studies of the 3 selected metal tolerant isolates belonging to the genus *Psychrobacter, Planococcus,* and *Halomonas* indicated the presence of diverse genes encoding for metal resistance, multi-drug/antibiotic resistance, and stress tolerance. It was noted that the metal resistant genetic elements- *merA, merR, czcD,* and *arsC* involved in the regulation and detoxification of metals Hg, Cd, Zn, Co, and As were present in all the three bacterial genomes. In addition, genes encoding manganese uptake and export (*mnt*), zinc uptake, transport and regulation (*znu, zup, zur*), molybdenum transport (*mod*), chromium resistance (*chr*), transcriptional regulatory proteins to regulate increasing levels of copper and silver (*cus*) and superoxide dismutase (*sod*) were present in one or more of the genomes studied. The presence of different efflux pump mediated multi-drug-resistant genes belonging to RND (resistance-nodulation-division) family transporters was observed in all three genomes. This suggested the presence of efflux pump genes as a common strategy in Arctic bacteria to export antibiotics and other noxious substances across the cell surface to reduce their intracellular load. All three bacterial genomes accounted for the presence of cold stress-related genes (*csp*) indicative of their Polar signature.

  To conclude, our study suggests the added value of examining bacterial communities to better understand and trace the transport of meltwaters from their glacial source to the oceans, since we could observe a gradual loss in the glacier

associated taxa on reaching the fjord system. The significant differences in the bacterial community composition between the glacier and the fjord ecosystem suggest the unique nature of these systems which in turn is influenced by the associated environmental parameters. Our polyphasic approach following both culture-dependent and culture-independent techniques along with the detailed metabolic profiling of the sediment bacterial communities contributes to a better understanding of their taxonomic and metabolic diversity which could be a base to understand bacterial-environmental interactions and underlying ecological processes. This study is the first of its kind, to give a clear indication of the potential of Arctic glacio-marine bacteria to tolerate metals that are of ecological significance to the Arctic (mercury, cadmium, lead, nickel, cobalt, zinc, and manganese) along with their detailed genomic analysis. Our findings strongly suggest that the selected metal tolerant bacterial isolates are suitable candidates exploitable for bioremediation applications in Polar Regions since they could efficiently accumulate the different metals tested in the study. The detailed genomic studies of the selected bacterial species indicated the presence of well-developed metal resistant and antibiotic/multi-drug resistant genetic elements with potential efflux pump mechanisms active in all three genomes substantiating their potential role for bioremediation.

Future scope of the study:

Future work envisages more focused experiments, quantifying the bacterial activity, gene abundance, and protein expression in response to the different metal enrichments. A whole genome-metagenome-based study on the different glacio-marine environmental samples is proposed for understanding the highly prevalent functional pathways from the reconstructed genomes which make them fit to tolerate the different environmental stresses and thrive in the cold Polar conditions. This would ultimately lead to novel insights into the metal resistance pathways in Arctic bacteria and their potential role in trace element cycling in the Arctic.

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Annexure I

Data Availability statement

All the bacterial sequence data has been deposited to NCBI database.

Genbank Submission details for 16S rRNA gene sequences of bacterial strains:

Accession numbers:

- MH478303 - MH478317, MH478329 - MH478331, MH478333 - MH478335, MH478337 - MH478341, MH478345 - MH478347, MH482212 - MH482349, and MK367606 - MK367610.
- MN080149 - MN080222, MT309496 - MT309525.
- MZ311755 - MZ311852.

Sequence Read Archive (SRA) database submission details for raw DNA nucleotide sequence datasets from Amplicon sequencing:

Accession number:

- SRP150318
- PRJNA475645

Drfat genome sequence submission details:

- The draft genome sequence of *Psychrobacter* sp. HM11 was deposited in the DDBJ/EMBL/GenBank database under the Accession number JAKUDE000000000.
- The draft genome sequence of *Planococcus* sp. HM12 was deposited in the DDBJ/EMBL/GenBank database under the Accession number JAKVTG000000000.
- The draft genome sequence of *Halomonas* sp. HM116 was deposited in the DDBJ/EMBL/GenBank database under the Accession number JAKVTW000000000.
- The draft genome sequence of *Phenylobacterium* sp. 20VBR1^{T} was deposited in the DDBJ/EMBL/GenBank database under the Accession number JAGSGD000000000.

Annexure II

List of Publications from the Thesis

1. **Thomas, F. A.**, Sinha, R. K., Krishnan, K. P., 2020. Bacterial community structure of a glacio-marine system in the Arctic (Ny-Ålesund, Svalbard). ***Science of The Total Environment***. *718*, 135264. https://doi.org/10.1016/j.scitotenv.2019.135264
2. **Thomas, F. A**., Mohan, M., Krishnan, K. P., 2021. Bacterial diversity and their metabolic profiles in the sedimentary environments of Ny-Ålesund, Arctic**.** ***Antonie van Leeuwenhoek*.** DOI:10.1007/s10482-021-01604-9
3. **Thomas, F. A**., Sinha, R. K, Hatha, A. A. M., Krishnan, K. P., 2022. *Phenylobacterium glaciei* sp. nov., isolated from Vestrebroggerbreen, a valley glacier in Svalbard, Arctic. ***International Journal of Systematic and Evolutionary Microbiology***. DOI: 10.1099/ijsem.0.005375

List of Other Publications

1. Jain, A., Krishnan, K.P., Begum, N., Singh, A., **Thomas, F.A**., Gopinath, A., 2020. Response of bacterial communities from Kongsfjorden (Svalbard, Arctic Ocean) to macroalgal polysaccharide amendments. ***Marine Environmental Research***. 155, 104874. https://doi.org/10.1016/j.marenvres.2020.104874
2. Jain, A., Krishnan, K.P., Singh, A., **Thomas, F.A**., Begum, N., Tiwari, M., Bhaskar, V.P. and Gopinath, A., 2019. Biochemical composition of particles shape particle-attached bacterial community structure in a high Arctic fjord. ***Ecological Indicators***. *102*, 581-592. https://doi.org/10.1016/j.ecolind.2019.03.015
3. Sinha, R.K., Krishnan, K.P., **Thomas, F.A**., Binish, M.B., Mohan, M. and Kurian, P.J., 2019. Polyphasic approach revealed complex bacterial community structure and function in deep sea sediment of ultra-slow spreading Southwest Indian Ridge. ***Ecological Indicators***. *96*, 40-51. https://doi.org/10.1016/j.ecolind.2018.08.063

4. Sinha, R. K., Krishnan, K. P., Singh, A., **Thomas, F. A**., Jain, A. and Kurian, P. J., 2017. *Alteromonas pelagimontana* sp. nov., a marine exopolysaccharide-producing bacterium isolated from the Southwest Indian Ridge. ***International Journal of Systematic and Evolutionary Microbiology***. 67(10), 4032-4038. https://doi.org/10.1099/ijsem.0.002245

5. Potdar, S., Daniel, D. K., **Thomas, F. A**., Lall, S. and Sheeba, V., 2018. Sleep deprivation negatively impacts reproductive output in Drosophila melanogaster. ***Journal of Experimental Biology***, jeb-174771. https://doi.org/10.1242/jeb.174771

Conferences in which the findings from the thesis research were presented

1. **Thomas, F.A.**, Mohan, M., Krishnan, K.P, Bacterial community structure and their metabolic profiles in the sedimentary environments of Ny-Ålesund, Arctic - oral presentation under the session " B11B: Biogeochemical Responses of the Cryosphere to Environmental Change I ” in **AGU 2021 Conference** (Participated Online, 13 December, 2021)

2. **Thomas, F.A.**, Krishnan, K.P, Insights into metal tolerant bacterial diversity associated with a glacio-marine system in Ny-Ålesund, Arctic- oral presentation under the session "Microbiomes and Biogeochemical Processes along Geographic and Environmental Gradients in the Circumpolar North (2)” in **Arctic Science Summit Week 2021 Conference** (Online Conference, Portugal, 19-26, March, 2021)

3. **Thomas, F.A.**, Sinha, R.K., Krishnan, K.P, Bacterial community structure in two fjord systems of Spitsbergen Arctic, Poster presentation for **International Conference on Frontiers in Marine Science - Challenges and Prospects (MARICON 2019)**, at Cochin University of Science and Technology (16-20, December, 2019)

4. **Thomas, F.A.**, Krishnan, K.P., Singh, A., Jain, A., Sinha, R.K., Mohan, M, Bacterial diversity along a glacio-marine transect in Ny-Ålesund, Arctic - Poster presentation for Young Polar Scientist Meet (YPSM) at **National Conference on Polar Sciences 2019** organized by NCPOR, Goa, India (20-22, August, 2019).

Science of the Total Environment 718 (2020) 135264

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Bacterial community structure of a glacio-marine system in the Arctic (Ny-Ålesund, Svalbard)

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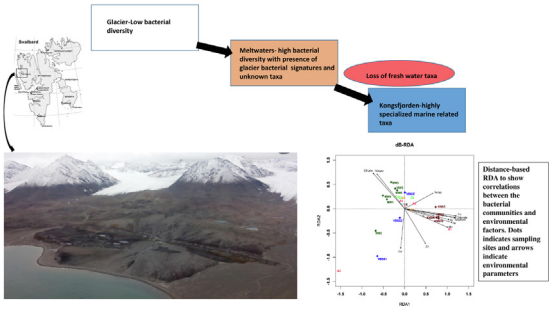
HIGHLIGHTS

- Significant difference in the bacterial diversity of glacio-marine system in Arctic.
- MWs exhibited highest diversity index while glacier snow exhibited the lowest.
- Kongsfjorden community dominated by marine taxa known for organic matter degradation.
- Nutrients correlated with the MW bacterial community.
- Trace metals, ions and temperature correlated with the fjord bacterial community.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 7 June 2019
Received in revised form 25 October 2019
Accepted 27 October 2019
Available online 23 November 2019

Editor: Sergi Sabater

Keywords:
Bacterial community
Svalbard
Glacier
Melt water
Kongsfjorden
High-throughput amplicon sequencing

ABSTRACT

The bacterial community composition of a valley glacier in Svalbard, its pro-glacial channels, and the associated downstream fjord ecosystem was investigated so as to figure out the degree to which down-slope transport of microbes from the glacier systems along a hydrological continuum impose an effect on the patterns of diversity in the fjord system. A combination of culture based and high-throughput amplicon sequencing approach was followed which resulted in significant variation ($R = 0.873$, $p = 0.001$) in the bacterial community structure between these ecosystems. Dominance of sequences belonging to class β-Proteobacteria was seen in the glacier snow, ice and melt waters (MW) while a relatively higher abundance of OTUs belonging to α-Proteobacteria and Verrucomicrobiae demarcated the fjord waters. Similarity percentage (SIMPER) analysis of the Operational Taxonomic Units (OTUs) showed that OTU 1,105,280 (9.15%) and OTU 331 (6.5%) belonging to *Burkholderiaceae* (β-proteobacteria) and OTU 101,660 (5.76%) and OTU 520 (5.07%) belonging to *Rhodobacteraceae* (α-proteobacteria) contributed maximum to the overall dissimilarity between the sampling sites. The bacterial community from the MWs were found to be true signatures of the glacier ecosystem while the Kongsfjorden bacterial fraction mostly represented heterotrophic marine taxa influenced by warm Atlantic waters and presence of organic matter. Significant presence of unknown taxa in the MWs suggests the need to study such unexplored, transient niches for a better understanding of the associated microbial processes. Among the various environmental parameters measured, nutrients (NO_3^- and SiO_4^{2-}) were found to exhibit strong association with the MW bacterial community while temperature, trace metals, Cl^- and SO_4^{2-} ions were

Abbreviations: MW, meltwater; VB, Vestrebroggerbreen.

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https://doi.org/10.1016/j.scitotenv.2019.135264
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found to influence the fjord bacterial community. The significant differences in the bacterial community composition between the glacier and the fjord ecosystem suggest the unique nature of these systems which in turn is influenced by the associated environmental parameters.

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1. Introduction

The Arctic archipelago of Svalbard in the North Atlantic (74°to 81°N) comprise an area of 62,248 km^2, of which ~57% is glacierized with a mix of cirque and valley glaciers, ice fields and ice caps (Nuth et al., 2013). Svalbard is one of the places in High Arctic where rapid changes in landscape forms have occurred due to deglaciation which in turn is a consequence of Arctic climate warming (Kim et al., 2017). Many of the Svalbard glaciers are reported to be rapidly retreating over the past 30 years with an average decline in the glacierized area of ~80 km^2 a^{-1} indicating a total reduction of 7% (Nuth et al., 2013). The glaciers present in the Svalbard archipelago are mostly polythermal and many exhibit surge-type behavior. The low flow velocities and the polythermal structure of the Svalbard glaciers are important in determining the glacial drainage system (Hagen et al., 2003). The land terminating/valley glaciers impart unique footprints on the downstream ecosystems such as the rivers and the streams and the nearshore marine ecosystems by causing shifts in biogeochemical and sediment fluxes (Hawkings et al., 2016), biodiversity loss (Brown et al., 2007; Jacobsen et al., 2012), and altered food web dynamics (Gutiérrez et al., 2015).

Glaciers harbor highly diverse microbial assemblages within their habitats such as snow, surface ice, cryoconite holes, englacial systems and the interface between ice and overridden rock/soil (Anesio et al., 2017). These microbes might have probably dispersed from close and distant locations with dispersal vectors such as terrestrial dust or aerosols (Margesin and Miteva, 2011) and have the capacity to thrive wherever there is enough liquid water to sustain their activity. During summer, when there is a ready supply of meltwater across much of the glacial cryosphere, microorganisms soon become active in cryoconite holes, supraglacial streams, moraines, and snowpacks. Microbial communities are majorly responsible for changes in the biogeochemistry of runoff that have been identified following the emergence of ice and snow melt from the glacier surface as well as the glacial bed (Hodson et al., 2008). The degree to which downslope transport of microbes from the glacier systems along a hydrological continuum contribute to varying patterns of microbial diversity and their ecological dynamics is a lacuna in Norwegian High Arctic region. Furthermore, understanding the microbial communities present in these systems is of great importance to gauge the ecosystem response to global warming (Blaud et al., 2015).

The bacterial and archaeal community play key roles in driving the biogeochemical cycling of nutrients, thereby facilitating growth and succession by subsequent trophic levels. The bacterial diversity studies which were conducted in the different Svalbard ecosystems such as the glacier snow cover (Larose et al., 2010; Lutz et al., 2017; Hell et al., 2013), glacier forefields (Vardhan Reddy et al., 2009; Kim et al., 2017; Ntougias et al., 2016), proglacier lakes (Wang et al., 2016), and Kongsfjorden (Sinha et al., 2017a, 2017b; Zeng et al., 2013; Jain and Krishnan, 2017a, 2017b) using the retrievable techniques as well as the next generation data have significantly contributed to the existing knowledge on bacterial community composition and their functional dynamics in these ecosystems. However, a single study integrating the glacier ecosystem to its pro-glacial networks and the associated downstream marine ecosystem with special emphasis on the spatial distribution of bacterial community has not been undertaken.

The continuous hydrological network connecting the land terminating glacier to the fjord through the different melt water channels conduct nutrients, sediments, organic and inorganic debris along with microbial load to the downslope environments (Hop et al., 2002). However, there could be a gradual decrease in the bacterial diversity on reaching the fjord due to the loss of many fresh water associated taxa introduced from upstream environments combined with the increase in relative abundance of a few specific taxa that are best adapted for marine ecosystem. Therefore, a study area was chosen encompassing the accumulation and ablation snow and snout ice from the Vestrebroggerbreen glacier (land terminating glacier), melt waters from the snout region of glacier traversing along a 4 km transect up to the Kongsfjorden system and fjord waters from the point where meltwater channels are emptying, along with outer fjord sample which is the seaward part of the fjord and inner fjord samples where the fjord has direct influence from the different tidewater glaciers. To understand whether the bacterial community composition exhibit significant variations within these systems and whether the downstream fjord bacterial community is being structured by the glacier and pro-glacier systems, we have (1) compared the bacterial community structures in VB glacier snow and ice, associated MW channels (Bayelva river) and Kongsfjorden ecosystem through culture based and high-throughput sequencing approach, and (2) elucidated the role of environmental factors such as temperature, major anions (Cl^- and SO_4^{2-}), nutrients (NO_3^-, NO_2^-, SiO_4^{2-} and PO_4^{3-}) and trace metals (Mn, Fe, Ni, Co, Cu and Zn) in structuring the bacterial community in each of these sampling sites. This would help us in understanding the similarities and differences that exist in the bacterial diversity pattern along the entire transect of this glaciomarine system in Svalbard as controlled by dispersal vectors and associated environmental factors.

2. Materials and methods

2.1. Sampling sites

The studied area is located in the west coast of Spitsbergen, in Svalbard archipelago (Fig. 1A and B). Vestrebroggerbreen (VB) glacier is a 4.7 km^2 long polythermal valley glacier in Ny-Ålesund situated between 78°53′26.9″N–78°55′14.5″N and 11°38′50.3″E–11°47′23.3″E (Fig. 1A). The total glacierized area is over 17 km^2. Bayelva river is the principal meltwater channel (~4 km long) draining the Brøggerbreen valley glacier into Kongsfjorden (Fig. 1A). Kongsfjorden is a relatively small fjord (26 km long and 4–10 km wide), situated between 78°04′ N–79°05′ N and 11°03′ E–13°03′ E (Fig. 1B). The outer fjord is influenced by the oceanographic conditions and the inner fjord is influenced by large tidal glaciers (Svendsen et al., 2002).

2.2. Sampling strategy

The samples were collected from different geographical transects of VB glacier and Kongsfjorden during Indian Arctic expedi-

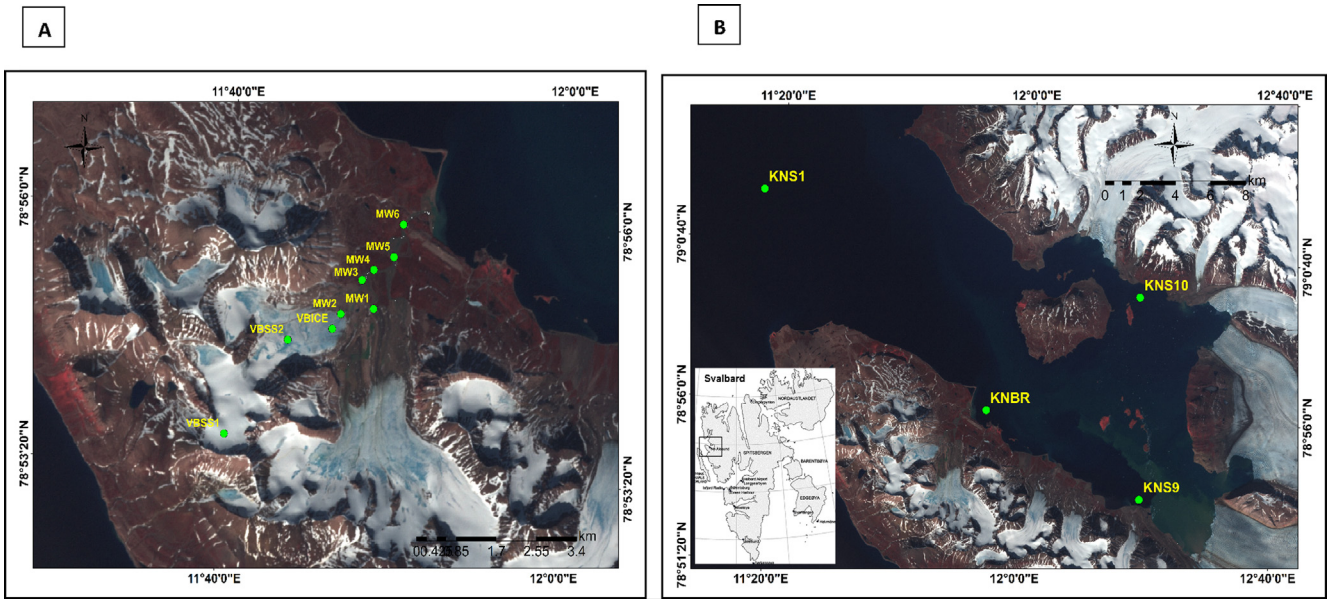

Fig. 1. (A). Map of Vestrebroggerbreen (VB) glacier and associated Melt water channels. Snow samples were collected from the accumulation (VBSS1) and ablation (VBSS2) zones of the VB glacier. Ice samples were collected from the VB snout region (VB Ice). Melt water samples were collected from points starting from the glacier terminus towards the Kongsfjorden and along the Bayelva River (MW1 to MW6). (B). Samples from Kongsfjorden were collected from two stations located at inner fjord (KNS9 & KNS10), outer fjord (KNS1) and from the opening of the Bayelva River into the fjord (KNBR). Map of Svalbard indicating the study area is given in the inset. (https://earthexplorer.usgs.gov/).

tion (August-September 2017). Snow samples were collected from the accumulation as well as the ablation regions of the VB glacier while the ice was collected from the snout region (in sterile Nasco sampling bags, Himedia). The snow and ice samples were collected in triplicates so as to ensure enough biomass for DNA extraction. These samples were then thawed at 4 °C (~22–24 h) and the triplicates were mixed to get a single representative VBSS1 snow accumulation sample, VBSS2 snow ablation sample and VB ice sample. Melt water samples (MW1-MW6) were collected from the associated pro-glacial channels and Bayelva River which were starting from the VB snout region up till the opening into Kongsfjorden waters, at every 700–750 m interval. We have collected water samples from the Kongsfjorden at the site where the MW channels are opening into the fjord (KNBR). The outer fjord station (KNS1) as well the 2 inner fjord stations (KNS9 and KNS10) water samples were also collected, so as to have a comparison with the KNBR waters to see whether the KNBR station is more influenced by the MW input in terms of its bacterial community from the VB glacier or is more similar to the other fjord samples (Fig. 1(a) and (b)). The melted snow and ice samples (~2 L from each of the triplicate) and MW samples (~2 L) from the pro-glacial channels were filtered through 0.2 μm GTTP filter papers (Merck Millipore, Germany) and were stored at −80 °C until nucleic acid extraction for bacterial community analysis. Sterile ultrapure water (MilliQ) was used as the filtration blank and was processed in the similar manner as the samples. On-site measurement of Total dissolved solids (TDS), temperature, and dissolved oxygen in the MWs were carried out using a Waterproof Portable meter (Cyberscan series 600). In-situ measurement of temperature, salinity and dissolved oxygen in Kongsfjorden was carried out using CTD profiler (SBE 19 plus V2, Sea Bird Electronics, USA). Water samples from Kongsfjorden were collected from the depth of chlorophyll maxima (10–50 m) using Niskin bottles and processed for bacterial community analysis as detailed above.

For the analysis of nutrients and ions, samples were stored (100 mL) in frozen state in polypropylene bottles. For the analysis of total organic carbon (TOC), samples were collected (25 mL) in acid washed (using 0.5% ultrapur HNO_3, Merck Millipore, Germany) and combusted (450 °C, for 4 h) glass vials and stored at −20 °C. For trace element analysis, samples were acidified with suprapur HNO_3 (Merck Millipore, Germany) and stored until further processing. All the samples were transported in frozen state to the home laboratory at NCPOR for the analysis.

2.3. Chemical analysis

The dissolved nutrients (NO_3^-, NO_2^-, SiO_4^{2-} and PO_4^{3-}) were measured using Seal AA3 analytical auto-analyzer following standard colorimetric methods (Grasshoff, 1983) with an accuracy of ±1% standard deviation with R^2 value of 0.999. Total organic carbon analysis was carried out by the high temperature combustion method (Hansell and Peltzer, 1998) using TOC-TN Analyzer (Shimadzu TOC-L_{CPH}). The instrument was calibrated using reagent grade potassium hydrogen phthalate as the organic carbon calibration standard and regular blank checks were done using deionized water (Milli-Q). Inorganic anions (Cl^-, and SO_4^{2-}) were measured using a Dionex ICS-1100 Ion chromatography system with a DS6 heated conductivity cell detector. A mixture of sodium carbonate (Na_2CO_3) and sodium bicarbonate ($NaHCO_3$) was used as the eluent and the sample injection volume was 25 μL as per the instrument protocol.

Trace elements present in the samples were analyzed by ICP-MS (Thermo iCAP Q ICP-MS with CETAC ASX-520 autosampler). Continuous calibration was performed with standard and blank solutions during the course of measurements. Standard solutions were prepared in 1% HNO_3 using ICP-multielement standard (30 elements) solution VI (Merck Millipore, Germany). The sample preparation was done following Yuan et al. (2004) and Link et al. (1998). The fjord water samples were diluted to 25 times so as to avoid the interference with the salts while the snow, ice and MW samples were taken without dilution.

2.4. Total bacterial counts and isolation of heterotrophic bacteria

For the analysis of total bacterial cell counts, water samples (10 mL) and melted snow and ice samples collected from all sam-

pling locations were fixed with buffered formaldehyde (2% final concentration) and stored in dark at 4 °C. Total bacterial counts in the samples were enumerated using a modified method of Porter and Feig (1980) after staining with 4, 6-diamidino-2-phenylindole (DAPI) (DAPI, 20 μL of 1 μg ml^{-1} working solution per ml). The stained cells were filtered through black 0.22 μm-pore-size polycarbonate membrane filters (Nucleopore Track-Etch Membrane, Whatman, Maidstone, UK) and counted using Leica DM6 B microscope with the aid of the DAPI filter (Excitation 325–375 nm and Emission 435–485 nm). Counts were made from minimum of 20 randomly chosen microscopic fields from each sample to obtain a reliable mean.

Culturable heterotrophic bacteria from the glacier snow, ice, MWs and fjord samples were enumerated by spread plate method. A 100 μL of sample suspension was directly plated onto solid R2A media (1/2 strength), Antarctic Bacterial Medium, Tryptone Soy Agar (TSA) (1/10th strength), Luria Bertani medium (1/2 strength), Actinomycete isolation agar, Zobell Marine Agar (ZMA) medium (1/4th strength) and Artificial Seawater (ASW) salts broth agar medium with 0.1% yeast and peptone. The plates were incubated at 4 °C and 20 °C for 2–3 weeks. Colonies with unique morphological features were isolated and sub-cultured to obtain pure cultures.

2.5. Rep-PCR analysis, PCR amplification of the 16S rRNA gene, sequencing and phylogenetic analysis

Total bacterial genomic DNA was extracted from the pure bacterial cultures using ChargeSwitch gDNA mini bacteria kit (Invitrogen, Carlsbad, CA, USA). The purity of the DNA extracts was verified by gel electrophoresis on 1% agarose. Rep-PCR amplification based fingerprinting technique was used with the BOX A1R primer (5′- CTACGGCAAGGCGACGCTGACG-3′) to eliminate bacterial species subtypes and to select unique bacterial isolates for further studies (Rademaker et al., 1998). Rep-PCR was carried out in a final reaction volume of 25 μL, containing, 0.3 pM/μL of primer, 12.5 μL of AmpliTaq Gold 360 Master Mix (Applied Biosystems, CA, USA) and 2.5 μL of 360 GC enhancer (Applied Biosystems, CA, USA) and template DNA with a concentration of ~20 ng/μL in a thermocycler (BioRad CFX 96, Applied Biosystems, USA) with the following conditions: initial denaturation at 95 °C for 7 min followed by 29 cycles of 1 min at 94 °C, 1 min at 53 °C and 8 min at 72 °C, and a final extension of 10 min at 72 °C. Amplification was confirmed by electrophoresis in 2% agarose gel (Yang and Yen, 2012).

Isolates with unique banding patterns in the Rep-PCR gel were further chosen for 16S rRNA gene amplification and sequencing as per Sinha et al (2017a). The obtained sequences were assembled using BioEdit sequence alignment editor version 7.0.5.3 (Hall et al., 1999) and the obtained consensus contig was screened for chimera sequences using CHIMERA detection program (http://rdp8.cme.msu.edu/cgis/chimera.cgi) and were removed from the further analysis. The 16S rRNA gene nucleotide sequences having greater than 1200 bp were further analyzed in EZbiocloud server to identify their phylogenetic affiliation against already known validly published type strain species (Yoon et al., 2017). A total of 172 identified 16S rRNA gene sequences belonging to the isolated unique strains were deposited in GenBank under the accession numbers MH478303-MH478317, MH478329-MH478331, MH478333-MH478335, MH478337-MH478341, MH478345-MH478347, MH482212-MH482349 and MK367606-MK367610.

2.6. DNA extraction and metagenomic library preparation for amplicon sequencing

Total DNA was extracted from the previously frozen 0.22 μm pore-size polycarbonate membranes filters using DNeasy Power Soil kit (Qiagen, USA) according to the manufacturer's protocol. The quantity of the DNA was measured using the Biospectrometer (Eppendorf) (Supp Table S3) and the quality of the eluted DNA samples were checked on gel electrophoresis by using 1% agarose gel. The DNA samples were sent to Agrigenome Pvt Ltd (India) for amplification of V3–V4 region of the bacterial 16S rRNA gene using Pro341F/Pro805R primer (Takahashi et al., 2014). 16S rRNA PCR negative controls were maintained during the sequencing reaction so as to ensure the purity of the PCR reagents used. However, negative controls (both milliQ filtration control and 16S rRNA PCR negative control) were not sequenced for this study and, as such, we were not able to directly remove possible contamination brought by the DNA extraction kit. The amplified products were added with multiplex identifier tags and template-specific nucleotide primers for each sample and sequenced under HiSeq platform 2500 (Illumina, U.S). The raw DNA nucleotide sequence datasets were deposited into the NCBI Sequence Read Archive (SRA) database under the accession number: SRP150318.

2.7. DNA sequence data curation and bioinformatics analysis

Further downstream processing of raw DNA sequence datasets were carried out by the methods described in Caporaso et al (2010) with the following modifications. Sequences belonging to 16S rRNA gene primers and multiplexing adapters were removed from the raw sequence datasets by using Cutadapt (v.1.18) software (Martin, 2011). Quality of the reads were further checked using the FastQC program followed by merging the paired end reads using Seqprep program (v.1.3.2) (https://github.com/jstjohn/SeqPrep). The merged sequences were pooled and clustered into Operational Taxonomic Units (OTUs) using the open reference OTU picking protocol (Rideout et al., 2014). The clustering of reads into OTUs (at 97% sequence similarity) was performed against Greengene reference database (v.13_5) using Uclust program. In this step, singleton OTUs as well as OTUs whose representative (i.e., centroid) sequence couldn't be aligned with PyNAST were removed. Chimeric sequences were removed from further data analysis by using USEARCH 6.1 package (Edgar, 2010) in QIIME. Unique representative OTUs from each sample were further subjected to taxonomic classification. OTUs representing the chloroplasts and mitochondrial sequences were removed from the analysis by using QIIME software (Caporaso et al., 2010). The OTU counts were rarefied (depth of rarefaction − 208331, 10 iterations) for all downstream diversity analysis. Various alpha diversity indices were calculated using Shannon, Simpson, Chao1 and observed species metrics in QIIME software. Double hierarchial dendrogram and the heatmap depicting the relative abundance of the top 100 bacterial OTUs within the 13 samples was plotted using the multivariate statistical software Primer (PRIMER E) version 7. Venny 2.1 software (Oliveros, 2007) was used to generate Venn diagrams to demonstrate the number of site-specific OTUs and the common OTUs shared between the sites.

Although studies with low microbial biomass are expected to be more sensitive to contaminants (Glassing et al., 2016; Ruuskanen et al., 2018), we tested the impact of possible contaminants by comparing our NGS data to the known contaminants sequences from MOBIO PowerSoil DNA extraction kit and Qiagen blood and tissue kit (Glassing et al., 2016). Note that the 10 most abundant contaminants reported (*Methylobacterium, Oxalobacter, Veillonella, Prevotella, Propionibacterium, Streptococcus, Staphylococcus, Tumebacillus, Corynebacterium and Enhydrobacter*) were found in our samples except for *Tumebacillus* related OTUs, but their relative abundance was found to be <1% in all the samples analysed (Supp Table S5). Exceptions were observed wherein the OTUs closely associated with *Methylobacterium* was found to show abundance up to 4% in VBSS1 snow sample and 3.6% in VBSS2 sample. Simi-

larly *Streptococcus* related OTUs were found to represent up to 6% in VBSS2 snow sample. However, as the putative contaminant genera could plausibly be part of the environmental bacterial community and the identity of true contaminants are not known, they were not removed from the data.

Analysis of similarities (ANOSIM) was performed to test the significance of the differences observed between the three sampling sites (VB glacier, MWs and fjord) using PRIMER 7 software. Similarity percentage (SIMPER) analysis was used to calculate the percent contribution of bacterial OTUs to average dissimilarity between the sampling sites (Clarke et al., 1993) and one-way analysis of variance (ANOVA) test was performed to determine the significant environmental parameters influencing the sampling sites using PAST (v.3.21) software (Hammer et al., 2001). The relevance of environmental factors in explaining the distribution patterns of bacterial communities in different sampling sites was done by distance based Redundancy analysis (db-RDA using Bray Curtis dissimilarity) using the vegan package in R v.3.1.1 statistical software.

3. Results

3.1. Environmental characteristics of the glacier, melt waters and fjord waters

The sampling locations and the various environmental parameters analyzed in the study are summarized in Table 1. The highest average concentrations of NO_3^- (5.65 μM) and SiO_4^{2-} (6.53 μM) were observed in the MWs as compared to the glacier snow, glacier ice and fjord waters while the highest values for NO_2^- (1.35 μM) and PO_4^{3-} (3.97 μM) were found in the glacier ice. Maximum average concentration of TOC was observed in the Kongsfjorden waters (25.6 ± 4 ppm). MWs had an average TOC value of 0.84 ± 0.2 ppm while the glacier ice had TOC value of 0.59 ppm. The highest average concentrations of Cl^- (16.18 ± 6.4 ppt) and SO_4^{2-} (2.14 ± 0.9 ppt) ions were found in the fjord waters. The average concentrations of Cl^- and SO_4^{2-} ions in the MWs were 5.91 ± 3.9 ppm and 16.47 ± 3.9 ppm while in the glacier snow, it was found to be 5.9 ± 0.1 ppm and 1.48 ± 0.2 ppm respectively. The highest concentrations of trace metals such as Mn, Fe, Ni and Cu were observed in the Kongsfjorden waters as compared to the glacier snow, ice and MWs. Among the Kongsfjorden samples, the inner fjord water sample KNS10 was found to exhibit the highest concentrations of Mn (25.11 ppb), Fe (249.9 ppb) and Ni (24.39 ppb). VB snow sample (VBSS1) exhibited the highest concentration of Zn (566.6 ppb) and Co (3.64 ppb) as compared to the glacier ice, MWs and fjord water samples (Table 2). It was observed that with the exception of PO_4^{3-} and NO_2^-, all other parameters such as NO_3^-, SiO_4^{2-}, Cl^-, SO_4^{2-} and the trace metals, contributed to significant differences between the glacier (snow and ice), MWs and fjord samples (ANOVA, $p < 0.05$) (Table 3).

3.2. Bacterial enumeration and identification of culturable bacteria

Total Bacterial Count (TBC) observed in the samples ranged between 10^7-10^8 cells/L with the lowest (2.2–3.4 × 10^7 cells/L) and highest count (5.5–7.2 × 10^8 cells/L) recorded in the snow samples and Kongsfjorden waters respectively. A total count of 5.4 × 10^7 cells/L was observed in the glacier snout ice while 6.2–7.5 × 10^7 cells/L were recorded in the MWs (Table 1).

The total retrievable bacterial count in the Kongsfjorden waters was found to be 10^6 CFU/L while MWs shown to have total retrievable count within the rage of 10^4-10^6 CFU/L, which was followed by the VB glacier ice sample (10^3 CFU/L) (Table 1). The total retrievable bacterial count for the VB glacier ice and MW samples were calculated using the colony counts obtained on TSA plates (1/10th strength) while for the Kongsfjorden total retrievable count, ZMA plates (1/4th strength) were used. We were unable to retrieve any bacteria from the VB snow by direct plating as well as by enrichment in broth media even after prolonged period of incubation (3 months).

A total of 20 (11 from 4 °C and 9 from 20 °C incubation), 510 (278 from 4 °C and 232 from 20 °C incubation) and 180 (114 from 4 °C and 66 from 20 °C) bacterial isolates were recovered from glacier ice, MWs, and Kongsfjorden waters respectively. Based on finger print profile generated by rep-PCR, a total of 20 isolates from the glacier ice (11 isolates from 4 °C and 9 isolates from 20 °C incubation), 214 from the MWs (129 isolates from 4 °C and 85 isolates from 20 °C incubation) and 34 isolates from the Kongsfjorden waters (14 isolates from 4 °C and 20 isolates from 20 °C incubation) were selected for 16S rRNA sequencing. The 16S rRNA gene sequence data analysis revealed the taxonomic grouping of bacterial isolates into 5 species from the glacier ice, 40 species from the MWs and 16 species from the Kongsfjorden waters (similarity cut-off >98.7%, Kim et al., 2014). (Supp Table S1). Seventeen bacterial isolates that were retrieved from the glacier ice and MWs showed <98.7% similarity with the closest phylogenetic neighbor (Supp Table S1) and hence might represent novel taxa.

The bacterial isolates retrieved from the glacier ice, MWs as well as from the fjord water samples belonged to three phyla namely, Proteobacteria (with the classes α, β and γ-proteobacteria), Bacteroidetes and Actinobacteria. Proteobacteria was found to be the most frequently encountered phyla in the glacier ice (66.6% of the total isolates), MWs (82%) and fjord (97%) waters and among which α-proteobacterial class dominated the VB ice (75% of the total proteobacteria) while γ-proteobacterial class was found to be most abundant in MW samples (61.25% of total proteobacteria) and fjord water samples (87.6%) (Fig. 2). In the VB ice, β-proteobacteria contributed to 25% of the total proteobacterial population while in MWs their contribution accounted for 31.6%. In the fjord water samples, β-proteobacteria could not be recovered. The other phyla, namely Actinobacteria was represented by 8.33%, 8.62% and 2% of the total isolates in glacier ice, MWs and fjord samples, while Bacteroidetes represented 25%, 9.35% and 0.6% of the total respectively (Fig. 2).

The culture medium TSA (1/10th strength) and R2A (1/2 strength) yielded maximum retrieval of colonies from the glacier ice and MW samples as compared to the other media used in the study (Supp Tables S4 and S5). It was also noted that the majority of the isolates retrieved from the glacier ice and MW samples were pigmented (orange, red, pink, peach and yellow). Among the VB ice isolates, *Polymorphobacter glacialis* (α-proteobacteria) was found to be the dominant species (37.5% of the total isolates), followed by *Flavobacterium piscis* (16.6%) and *Herminiimonas fonticola* (14.5%). The Actinobacterial phylum was represented by a single genus *Cryobacterium* with 3 different species namely – *C. psychrotolerans, C. flavum* and *C. levicorallinum*. While, in the MWs, *Pseudomonas extremaustralis* (48% of the total) was found to be the most abundant bacterial species along with substantial contributions from *Polaromonas* (19.7% of the total) and *Flavobacterium* (8.6%). In the MWs, class γ-proteobacteria was represented by a single genus, *Pseudomonas* while a total of 7 genera were recovered within the class α-proteobacteria wherein the species *Sphingorhabdus planktonica* was the major contributor (38% of α-proteobacteria). β-proteobacteria was represented by 7 genera among which *Polaromonas eurypsychrophila* was the predominant species (72% of β-proteobacteria). Three genera represented the phylum Bacteroidetes wherein the major fraction was contributed by the genus *Flavobacterium* (98% of Bacteroidetes). Phylum Actinobacteria was represented by 13 different genera, of which *Glaciihabitans* and *Cryobacterium* appeared

Table 1
Details of sampling locations and their associated physico chemical parameters measure in the study.

Parameters	Glacier			Melt Water Channels						Kongsfjorden			
	VBSS1	VBSS2	VB Ice	MW1	MW2	MW3	MW4	MW5	MW6	KNBR (DCM = 10 m)	KNS1 (DCM = 30 m)	KNS9 (DCM = 10 m)	KNS10 (DCM = 10 m)
Latitude	78.8945°N	78.9114°N	78.91375°N	78.91761°N	78.9164°N	78.92436°N	78.92436°N	78.92678°N	78.93249°N	78.93472222°N	79.0354°N	78.8951°N	78.9931°N
Longitude	11.6695°E	11.7274°E	11.77069°E	11.81048°E	11.77842°E	11.79769°E	11.80883°E	11.82803°E	11.83587°E	11.905277°E	11.2836°E	12.3201°E	12.3°E
Temperature (°C)	−6.2	−13.2	1	0.9	1.9	1.3	0.4	1.3	0.9	4.5	6	4.2	3.2
DO(mg/L)	ND	ND	ND	7.82	8.9	8.01	7.8	7.711	7.92	8.5	6.75	5.6	6
Total Dissolved Salts (TDS)/ Salinity (ppm for MW, ppt for fjord)	ND	ND	ND	81.39	24.58	107.8	109.7	87.19	93.43	33	34.5	33	31
Nitrite (μM)	0.09	0.08	1.35	0.27	0.09	0.13	0.08	0.84	0.63	0.08	0.01	0.05	0.1
Nitrate (μM)	0.08	0.08	1.3	4.6	1.3	8.98	7.93	5.37	5.74	0.62	0.11	0.43	0.56
Total Phosphate (μM)	0.25	0.21	3.97	0.22	0.25	0.29	0.24	1.95	1.66	0.27	0.2	0.22	0.17
Silicate (μM)	0.41	0.44	4.61	6.66	4.61	8.67	4.19	6.72	8.31	0.83	0.64	0.75	0.79
Chloride (ppm for Glacier and MW, ppt for fjord)	5.82	5.99	2.08	2.91	9.99	2.59	11.3	6.14	2.55	18.11	19.6	20.4	6.65
Sulphate (ppm for Glacier and MW, ppt for fjord)	1.63	1.32	[a]	11.2	[a]	21.75	14.81	16.44	18.16	3.14	2.16	2.33	0.93
TOC (ppm)	ND	ND	0.59	0.52	0.89	0.71	0.92	0.88	1.1	23.8	25.7	31.1	21.7
TBC (cells/L)	2.2×10^7	3.4×10^7	5.4×10^7	6.2×10^7	7.5×10^7	7.4×10^7	6.7×10^7	6.3×10^7	6.5×10^7	5.5×10^8	7.2×10^8	6.1×10^8	6.6×10^8
THB 4 °C (CFU/L)	ND	ND	9×10^3	3.6×10^5	7×10^4	5.9×10^5	5×10^5	1×10^4	7×10^5	3.8×10^6	7.3×10^6	4.3×10^6	7.56×10^6
THB 20 °C (CFU/L)	ND	ND	6×10^3	3.16×10^5	5×10^4	3.7×10^5	3.9×10^5	2.6×10^4	1×10^6	1.6×10^6	4.6×10^6	3.3×10^6	6×10^6

ND – Not Determined.
[a] Below detection limit.

Table 2
Details of the trace element concentration from various sampling sites.

Station Details	Mn (ppb)	Fe (ppb)	Co (ppb)	Ni (ppb)	Cu (ppb)	Zn (ppb)
VBSS1	6.32	23.08	3.64	0.40	0.12	566.6
VBSS2	4.93	10.67	1.88	3.92	0.14	421.67
VB ICE	0.59	3.37	0.02	0.37	0.16	7.77
MW1	0.08	0.44	0.01	0.03	0.19	1.04
MW2	0.55	2.08	0.01	0.16	0.04	9.47
MW3	0.94	7.99	0.01	1.85	0.22	18.81
MW4	0.36	4.69	0.04	0.19	0.13	9.98
MW5	2.32	3.73	0.03	0.16	0.12	4.46
MW6	1.15	3.36	0.02	0.36	0.11	2.6
KNBR	10.14	105.14	0.79	9.99	3.3	321.89
KNS 1	8.71	75.32	0.68	6.8	6.31	355.22
KNS 9	8.41	113.85	0.32	5.55	3.66	483.72
KNS 10	25.11	249.9	0.99	24.39	3.19	84.09

Table 3
One way analysis of variance (ANOVA) F value and p value for the various chemical parameters.

	F value	p value
Anions		
Chloride	29.29	0.0000659
Sulphate	25.21	0.000124
Nutrients		
Nitrate	11.63	0.002457
Silicate	16.27	0.000717
Phosphate	1.08	0.3752
Nitrite	1.16	0.3526
Trace elements		
Mn	8.46	0.007079
Fe	12.70	0.001799
Co	4.87	0.033325
Ni	6.96	0.01279
Cu	33.44	0.0000372
Zn	6.28	0.01714

The critical value of F for the one way ANOVA is 4.102821. For the p values < 0.05 and F value > F crit (critical value of F), there exist significant difference between the sampling groups (VB glacier, MWs and Kongsfjorden waters). Underlined p values indicate the significance of the chemical parameters.

to be most dominant (Supp Table S1). Of all the isolates identified from the VB glacier ice and MWs, 52% isolates were retrieved by incubating the samples at 4 °C, 23% isolates were from 20 °C incubation, while 25% isolates identified had their representatives at both temperature incubations.

For the Kongsfjorden water samples, maximum retrieval of colonies could be observed on ZMA (1/4th strength) media (Supp Table S4). From the culture based study in the Kongsfjorden waters, we could observe the highest relative abundance of *Alteromonas stellipolaris* (58.8% of the total), along with significant contributions from *Psychrobacter* (10.3%) and *Loktanella* sp. (7%). A total of 5 genera were recovered within the class γ-proteobacteria wherein *Alteromonas stellipolaris* (69% of γ-proteobacteria) was the major contributor. Among the species representing α-proteobacteria, *Loktanella salsilacus* was found to be the dominant candidate (59% of α-proteobacteria). The Actinobacterial candidates belonged to 3 different genera while the phylum Bacteroidetes was represented by two genera. Two bacterial species, closely related to *Flavobacterium degerlachei* and *Salinibacterium xinjiangense* were retrieved from MWs as well as fjord samples (Supp Table S2). Among the culturable isolates identified from Kongsfjorden waters, 40% of the isolates identified were retrieved by incubating the samples at 4 °C incubation, 47% isolates were isolated from 20 °C incubation, while 13% isolates identified had their representatives at both temperature incubations.

3.3. Bacterial diversity and community composition using 16S rRNA gene amplicon sequencing

A total of 49,734 OTUs were identified from the total of 3,513,596 sequences among 13 samples analyzed in the present study. Sequences affiliated with phyla Proteobacteria, Bacteroidetes, Verrucomicrobia and Actinobacteria were common among all the sampling sites.

Irrespective of the sampling sites, Proteobacteria was the dominant phyla in terms of its relative abundance (38.5% – 99.6%), with the glacier snow samples exhibiting the highest abundance (99.6% in VBSS1 and 83.5% in VBSS2) (Fig. 3a). The glacier ice bacterial community was dominated by the phyla Proteobacteria (41.6%), Bacteroidetes (36.2%), Cyanobacteria (12.8%) and Actinobacteria (5.3%). In the MWs, we could observe the abundance of Proteobacteria, Bacteroidetes, Actinobacteria along with increased presence of Candidate Division TM7 and OD1. The phyla which were found exclusively in the MWs include Caldiserica, Chlamydiae, Candidate Division GN04, Candidate Division BHI80-139, Candidate Division Kazan-3B-28 and Candidate Division FCPU426. In the Kongsfjorden waters, we could observe a marked increase in the OTUs belonging to phyla Verrucomicrobia from the outer to the inner fjord waters (6.25–24.35%) which was substantially higher as compared to the glacier and MW samples (Fig. 3a).

Among the Proteobacterial OTUs, we could observe the dominance of sequences affiliated with β-proteobacteria in the VB glacier snow samples (89.3% in VBSS1 and 33.2% in VBSS2). The α-proteobacterial OTUs represented about 10–16% of the total in both the glacier snow samples while the γ-proteobacterial OTUs exhibited a higher abundance in the VBSS2 snow sample (33.9%) as compared to the VBSS1 snow (0.2%) (Fig. 3b). The glacier ice community was dominated by β -proteobacteria (19.2%), Flavobacteriia (19.1%), α-proteobacteria (15.6%), Synechococcophycideae (12.2%) and Cytophagia (9.7%) along with substantial contributions of Actinobacteria, Sphingobacteriia and γ-proteobacteria. In the MW samples, the most abundant proteobacterial sequences encountered was β-proteobacteria (24–75%), followed by γ-proteobacteria (0.3–21%), α-proteobacteria (2.2–9.3%) and δ-proteobacteria (0.5–6.5%). The bacterial classes such as Cytophagia, Flavobacteriia, Sphingobacteriia, Actinobacteria and TM7-1 were in higher abundance in the MWs as compared to the glacier samples. Some of the major bacterial orders in the glacier and MWs include *Burkholderiales, Sphingomonadales, Methylophilales Flavobacteriales, Actinomycetales* and *Cytophagales*. In the Kongsfjorden waters, class α- proteobacteria (34.5–57.2%), Verrucomicrobiae (6.2–24.3%), γ-proteobacteria (7.6–16%), Flavobacteriia (9–17.38%) and Acidimicrobia (1.8–13.4%) were found predominant (Fig. 3b). The dominant bacterial orders observed in the fjord waters include *Alteromonadales, Oceanospirillales, Rhodobacterales* and *Rickettsiales* (Fig. 3c).

In the present study, 7 genera namely, *Salinibacterium, Fluviicola, Flavobacterium, Sediminibacterium*, *Sphingomonas, Alteromonas* and *Pseudomonas* were found to be shared between all 3 sampling sites (VB glacier, MWs and fjord waters). While studying the abundance of dominant OTUs (Top 25), we could observe many of the OTUs were belonging to unknown genera of the members of *Burkholderiaceae* in the glacier snow samples followed by the genus *Sphingomonas, Methylobacterium,* and *Burkholderia*. In the glacier ice, the dominance of genera *Flavobacterium*, *Leptolyngbya*, *Polaromonas*, *Sandarakinorhabdus* and *Methylotenera* could be observed. OTUs belonging to the genera *Albidiferax, Flavobacterium*, *Leptolyngbya*, *Polaromonas*, *Methylotenera* and *Bdellovibrio* contributed to the major bacterial fraction in MW samples. While in the Kongsfjorden samples, the most abundant genera were *Sulfitobacter, Pelagibacter, Octadecabacter*, *Marimicrobium* and unknown genera belonging to *Verrucomicrobiaceae* along with

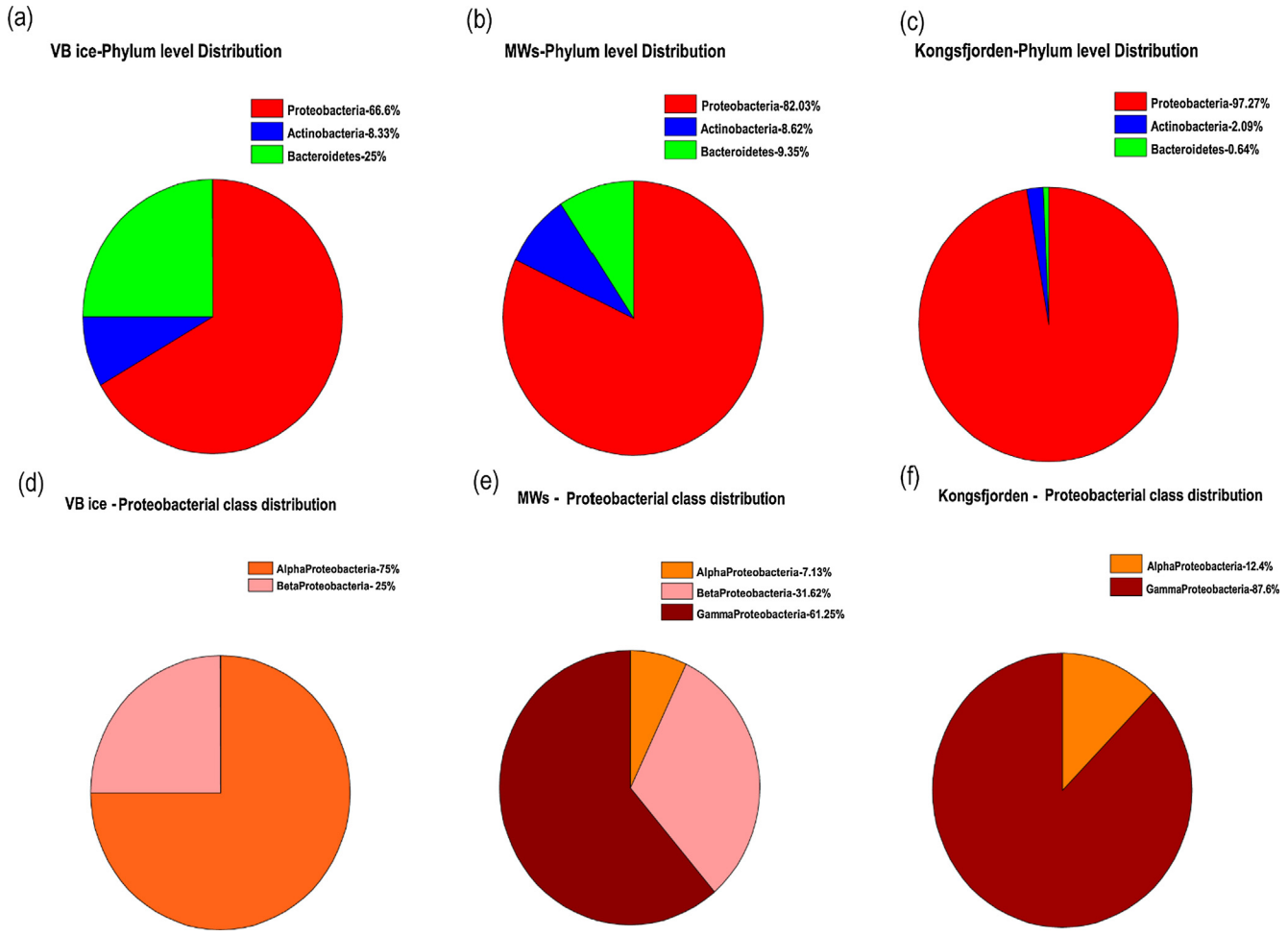

Fig. 2. Percentage composition and abundance of retrievable bacteria belonging to the phyla Proteobacteria, Actinobacteria and Bacteroidetes from VB ice (a), MWs (b) as well as from Kongsfjorden waters (c). The phylum proteobacteria which was found to be the dominant phylum among all the sampling sites were then further represented at their class level and the percentage abundance of each of the proteobacterial class for the VB ice (d), MWs (e) as well as for the Kongsfjorden (f) was plotted.

substantial contributions from *Loktanella*, *Fluviicola and Polaribacter*.

The comparison of the relative abundance of culturable bacterial species within the NGS dataset was also performed by blasting the sequences of bacterial isolates retrieved, against the 16S rRNA dataset of NGS created by using the blast version 2.7.1 installed within the linux system. It was observed that except for the species *Polaromonas eurypsychrophila,* which was the dominant β-proteobacterial representative in the retrievable fraction from the MWs (0–57.14% of the total bacterial isolates in the MWs), all other species retrieved contributed to <1% abundance of the total diversity obtained by NGS. Whereas, the relative abundance of *Polaromonas eurypsychrophila* was found to be 0.4–5.5% of the total in the MWs and 3.1% of the total in VB ice in the NGS results.

The dominance of the genus *Flavobacterium* from all the three sampling sites in both culture based and high throughput sequencing approach was noted. In the culture based study, Flavobacterium accounted for about 16% of the total bacterial genera retrieved from VB ice, up to 24.5% of the MW bacterial community, and up to 1.6% of the fjord bacterial community, while in the NGS based approach Flavobacterial genera accounted for about 17.8% of VB ice community, 4.35–20.9% of the total MW community and 0.35–1.96% of the fjord community. Similarly the presence of genus *Salinibacterium* could be observed in both MWs as well as in the fjord system in both our study approaches. The dominance of the genus *Polaromonas* from MWs and *Loktanella* from the fjord waters could also be observed from both culture based and high throughput sequencing study approaches.

The Shannon diversity index, a proxy for richness and evenness was found to be highest for the MW samples (6.26–10.67) with the highest value noted for MW5. The snow sample VBSS1 exhibited the least Shannon index value, 1.877 while VBSS2 had an index of 5.9. The Kongsfjorden waters exhibited Shannon index of 6.17–7.9, with the highest value noted for outer fjord station KNS1 (Table 4). The Chao_1 values and Observed species index values were found to be highest for the MW samples as compared to glacier and fjord samples (Table 4).

3.4. Differences in the bacterial community structure between the sampling sites and their correlation with environmental parameters

The hierarchial clustering analysis based on the relative abundance of the top 100 bacterial OTUs present within the 13 samples, yielded three clusters which corresponded to the three major sampling sites (Glacier, MWs and fjord waters), although we could see the clustering of VB ice with that of the MWs and not with the snow samples (Fig. 4). The Heat map plot depicted clear variation in the bacterial community compositions (OTUs) present within these three sites (Fig. 4). On analyzing the dominant OTUs from the heat map plot, it was observed that the OTUs belonging to *Burkholderiaceae* and *Sphingomonadaceae* were significantly abundant in both the glacier snow samples while the OTUs belonging

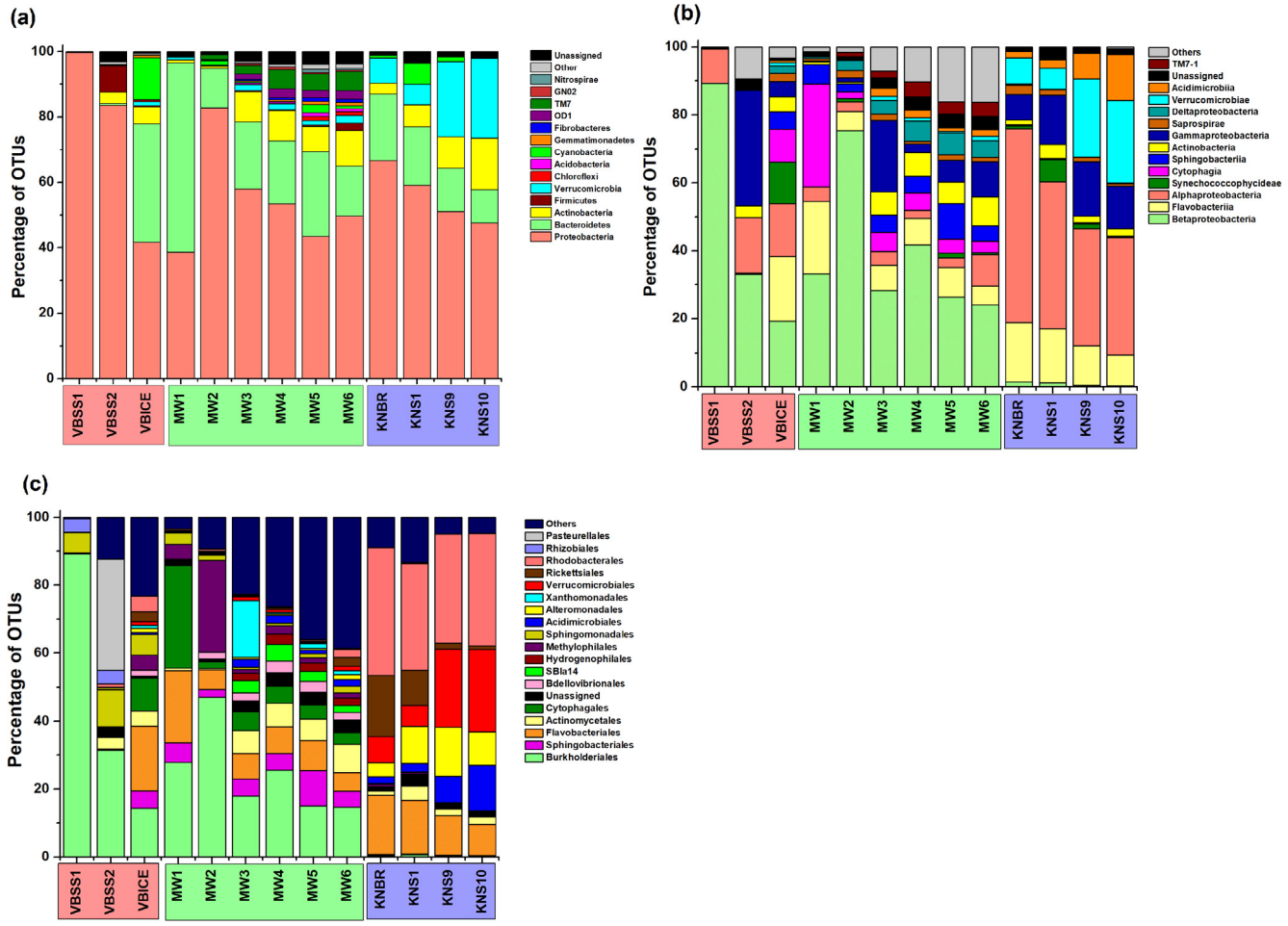

Fig. 3. The relative abundance of the (a) different phyla (b) different classes (c) different bacterial orders observed in the study. The sampling sites are marked in 3 different colors – Red – VB glacier snow and ice, Green – Meltwaters and Blue – Kongsfjorden. The category others include all the taxa below the abundance cut off 1.5%. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 4
List of obtained OTUs and associated diversity indices for each sampling site.

Sample	Total OTUs	Goods coverage	Shannon	Simpson	Chao1	Observed species
VBSS1	451	0.999	1.877	0.596	694.786	449
VBSS2	5184	0.998	5.907	0.933	5248.399	5154
VBICE	6588	0.991	8.247	0.990	9369.747	6359
MW1	7232	0.986	6.260	0.949	12,172.637	7148
MW2	6505	0.993	6.655	0.957	8522.597	6408
MW3	18,397	0.977	9.366	0.986	25,308.411	18,145
MW4	19,482	0.967	10.285	0.994	26,482.032	19,226
MW5	19,456	0.978	10.677	0.997	24,137.526	19,080
MW6	12,187	0.991	10.516	0.997	15,969.262	12,031
KNBR	7381	0.986	6.933	0.959	10,306.469	6795
KNS1	9880	0.989	7.915	0.979	10,260.769	8847
KNS9	5358	0.988	6.176	0.952	8237.557	5014
KNS10	6145	0.989	6.274	0.954	8156.120	5575

to *Rhodobacteraceae*, *Verrucomicrobiaceae* and γ-proteobacteria-clade OM60 dominated all 4 fjord samples. MWs exhibited the dominance of OTUs belonging to *Commamonadaceae* and *Flavobacteriaceae* which was even found to be dominant in the VB ice samples (Fig. 4).

An ANOSIM test (R = 0.873, p = 0.001: Bray-Curtis) also supported that the three sampling sites harbored significantly different bacterial communities (Table 5). The Venn diagrams (Fig. 5) demonstrated the number of site-specific OTUs and also the common OTUs shared between the sites. The Venn diagrams were plotted taking into consideration of all the OTUs present in at least 1 station within each sampling site (Fig. 5(a)) and also the OTUs that are present at all the stations within each sampling site (Fig. 5(b) and (c)). The Venn diagram with only VB ice component along with the shared OTUs of MWs and fjord (shared OTUs - the OTUs that are present at all the stations within each sampling site) demonstrated that 13% of VB ice OTUs were shared with all the MW stations while 8.6% of OTUs were shared with all the fjord stations (Fig. 5(c)). It was also observed from Fig. 5(c) that MWs shared 75% of their total shared OTUs with the VB ice

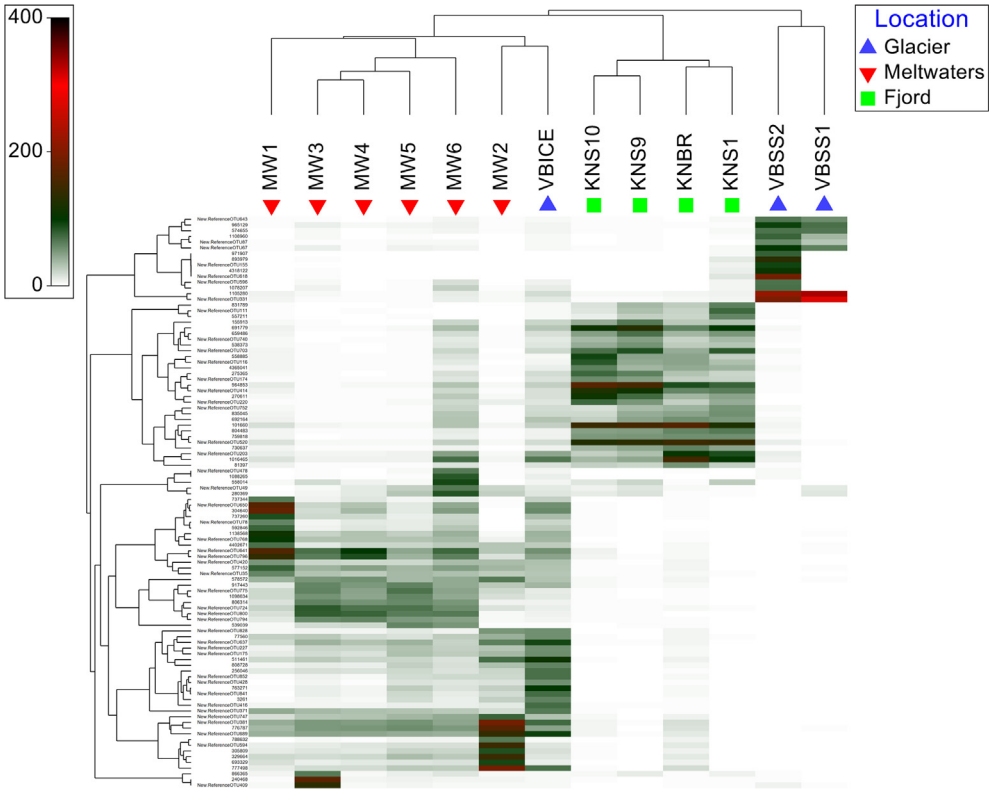

Fig.4. Double hierarchial dendrogram showing the bacterial distribution among the thirteen samples. The cluster dendrogram were standardized by square-root transformation and a Bray–Curtis dissimilarity matrix was generated. The heatmap plot depicts the relative abundance of the top 100 bacterial OTUs (variables clustering on the Y-axis) within each sample (X-axis clustering). The relative values for the bacterial OTU abundance are depicted by color intensity with the legend indicated at the top right corner of the figure respectively.

Table 5
Analysis of Similarity (ANOSIM) between sampling sites.

S.no	Sample Groups	R value	Level of significance
1.	Glacier, MW and Fjord pooled	0.873	0.001
2.	Glacier and MWs	0.722	0.012
3.	Glacier and Fjord	0.759	0.029
4.	MWs and Fjord	1	0.005

ANOSIM (testing whether two or more groups are significantly different) was calculated between all categories based on the abundance of total bacterial OTUs obtained. Each pairwise comparison of two groups was performed using 1000 permutations.

while only 35% of the fjord shared OTUs was common to that of VB ice.

The overall average dissimilarity between the VB glacier, MWs and the Kongsfjorden samples using SIMPER analysis was 95.23%. The OTU 1,105,280 (9.15%) and OTU 331 (6.5%) belonging to *Burkholderiaceae* and the OTU 101,660 (5.76%) and OTU 520 (5.07%) belonging to *Rhodobacteraceae* contributed maximum to the overall dissimilarity between the samples (Supp Table S2). The OTU 1,105,280 (*Burkholderiaceae*) contributed maximum to the significant difference between glacier and melt water (16.85%) as well as between glacier and marine fjord water (15.89%) while the OTU 101,660 contributed maximally to the significant differences between MW and fjord water (9.2%).

The db-RDA plot using Bray-Curtis dissimilarity clearly revealed the clustering of sampling sites in accordance with the relative abundance of different bacterial classes and various environmental parameters (temperature, nutrients, anions and trace metals) (Fig. 6). The close association of the nutrients (NO_3^-, SiO_4^{2-}) with the glacier ice and MW bacterial community and that of temperature, trace metals (Mn, Fe, Ni, Cu and Zn), Cl^-, and SO_4^{2-} ions with the fjord community could be clearly observed from the db-RDA plot.

4. Discussion

In the present study, we compared the bacterial diversity of Vestrebroggerbreen valley glacier along a transect with associated pro-glacial channels and the downstream marine ecosystem of Kongsfjorden using culture based and culture independent amplicon based high throughput sequencing techniques, so as to understand the degree to which the transport of microbes from the glacier system impose an effect on the patterns of diversity in the downstream ecosystem.

4.1. Unraveling the bacterial community structure using 16S rRNA gene amplicon sequencing

The dominance of sequences affiliated with β-Proteobacteria in the VB glacier snow (89.3% in VBSS1 and 33.2% in VBSS2) corresponds to the studies conducted previously in Arctic from glacier ice (Lutz et al., 2017) and glacier snow packs (Hell et al., 2013; Larose et al., 2010). Due to their rapid response to environmental fluctuations, β-Proteobacterial class is characterized as the r-strategists in aquatic as well as in terrestrial systems and are known for exploiting spatially and temporally variable resources which explain their potential to proliferate in transient niches such as the glacial snow (Hell et al., 2013). Among β-Proteobacteria, the sequences affiliated to the order *Burkholderiales* with family *Burkholderiaceae* were present in higher abundance in the snow samples. Majority of the members belonging to this order are reported to be metabolically and ecologically diverse, known for

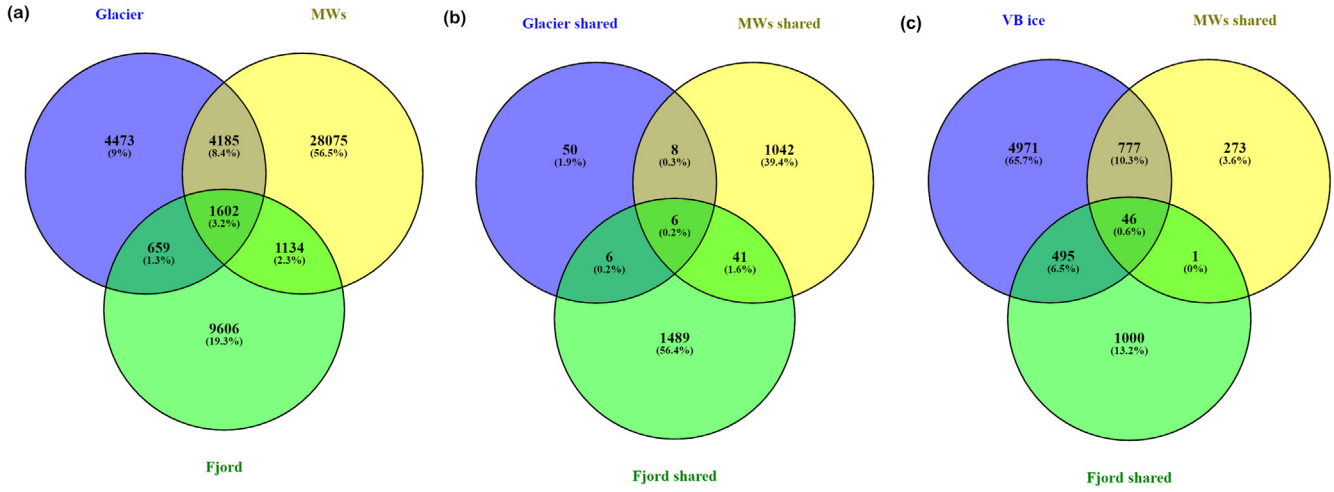

Fig. 5. (a) Venn diagram displaying the degree of overlap of bacterial OTUs among the three sampling sites taking into consideration of all the OTUs present in at least 1 of all the stations within each group, (b) Venn diagram displaying the degree of overlap of bacterial OTUs among the three sampling sites taking into consideration of only the shared OTUs present in each sampling group (shared OTUs means OTUs that are present in all the stations within each group), (c) Venn diagram displaying the degree of overlap of bacterial OTUs among the VB ice, shared OTUs of MWs and shared OTUs of Fjord waters.

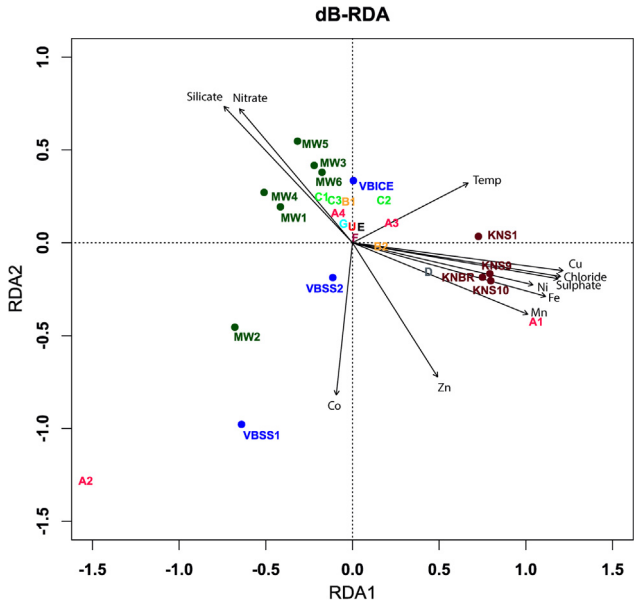

Fig. 6. Distance-based redundancy analysis to show correlations between the bacterial communities and environmental factors of the 13 samples from the three sampling sites. The arrows represent environmental factors measured such as nutrients, anions and trace elements. The 13 samples are labelled with unique sampling code and dots in: blue for VB glacier snow and ice, dark green for MWs and dark brown for Kongsfjorden waters. The length of the arrows indicates the relative importance of that environmental factor in explaining the variation in the bacterial communities, while the angle between each arrow and the nearest axis indicates the closeness of the relationship between each other. The bacterial classes are represented in different colors with unique alphabetical codes. The bacterial classes: A1 – α-Proteobacteria, A2 – β-Proteobacteria, A3 – γ-Proteobacteria and A4 – δ-Proteobacteria, B1 – Actinobacteria and B2 – Acidimicrobiia, C1 – Cytophagia, C2 – Flavobacteriia, and C3 – Sphingobacteriia, D – Verrucomicrobiae, E – Synechococcophycidiae, F – Saprospirae, G – TM7-1, U – Unassigned. The environmental parameters include Temp-temperature, Nitrate, Silicate, Chloride, Sulphate, and trace elements such as Mn-Manganese, Fe-Iron, Ni-Nickel, Co-Cobalt, Cu-Copper and Zn-Zinc. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

their ability to fix atmospheric nitrogen and utilize a wide range of organic compounds as carbon sources (Coenye and Vandamme, 2003). Burkholderiales have often been found to be dominant in sediments beneath glaciers and subglacial ecosystems (Skidmore et al., 2005) and are known to use organic matter eroded from glaciers and transported by glacial or melt water (Algora et al., 2015). Their presence in the glacier snow might be an indication for their significant contribution pertaining to the nutrient cycling within the glacier ecosystems (Algora et al., 2015). Among the top 25 OTUs analyzed from the snow samples, we could observe the abundance of genera *Sphingomonas* and *Methylobacterium*, along with the dominance of uncultured genera belonging to *Burkholderiaceae*. The dominance of sequences belonging to *Sphingomonas* in the top and middle layers of snow have been reported by Møller, et al. (2013) while the dominance of *Methylobacterium* spp. from ice core samples from Antarctica along with their ability to metabolize single carbon substrates to cope with the oligotrophic conditions in the ice has been reported by Antony et al. (2012). The low nutrient concentrations observed in the snow samples (Table 1) could explain the abundance of these genera which are capable of surviving such cold oligotrophic conditions.

The accumulation zone snow (VBSS1) community was dominated by the β-proteobacterial class while an increased presence of heterotrophic γ-proteobacterial class was noted in the ablation snow sample VBSS2 (33.9%). This could be possibly due to the deposition from terrestrial dust or aerosols (aeolian) or could be associated with the presence of supraglacial features such as cryoconites which provides dissolved nutrients and organic carbon, favoring the abundance of heterotrophic bacterial community (Xiang et al., 2009). The highly diverse VB glacier ice community dominated by OTUs belonging to the genera *Polaromonas* (β-Proteobacteria) and *Flavobacterium* (Bacteroidetes) can be linked to the higher nutrient concentrations observed in the glacier ice, compared to the snow samples (Table 1) which in turn can be due to the deposition of sediments, organic and inorganic debris on the snout ice through aeolian as well as post depositional events (Xiang et al., 2009). The higher abundance of OTUs belonging to the genus *Leptolyngbya* (Synechococcophycideae) in the VB ice can be related to the light rich conditions in the glacier surface and suggests that this bacterial group could be one of the common phototrophs in the glacier system sustaining the dynamics of heterotrophic bacterial community in the glacier and pro-glacier environment (Xiang et al., 2009).

The MW bacterial community was comparable to the glacier ice bacterial community wherein the major players where β and γ-proteobacteria along with significant contributions from the phyla Bacteroidetes with the classes Cytophagia, Flavobacteriia and Sph-

ingobacteriia. The presence of many candidate phyla exclusively in the MW samples is an indication that the pro-glacial channels although being transient niches support diverse and rare bacterial communities. The bacterial genera that were repeatedly encountered in the NGS analysis of MW samples included *Flavobacterium, leptolyngbya, Polaromonas,* and *Methylotenera* many of which could be traced back to the VB glacier snout ice community. The OTUs affiliated with the genus *Polaromonas* are known to oxidize a wide range of substrates in cold environments and recent genomic studies have revealed their alternative dormancy mechanism which may allow them to survive in extreme glacial environments and colonize recently deglaciated areas in higher abundance (Darcy et al., 2011). The *Flavobacterium* affiliated OTUs were previously noted from low salinity, cold environments such as polar lakes, streams, rivers and cold muddy soils (Bernardet and Bowman, 2006) while the cyanobacterial genus *Leptolynbya* are known for their significant ecological role in various microbiocenoses in coastal deglaciated zones (Komárek, 2007) and their photosynthetic activity could be critical in governing the pro-glacier dynamics. Likewise, the presence of methylotrophic bacteria belonging to the genera *Methylotenera* from the MWs is an indication for the variable functional potential of the bacteria associated with the transient MW ecosystem. The MW channels forms connecting link between the glacial processes and the downstream habitats and are subjected to daily and seasonal variability which includes changes in the stream discharge volume, temperature, sediment load, nutrient flux and redox conditions (Hotaling et al., 2017). Also, the time that the bacteria spend in the MW channels (residence time) is too short to establish a stable bacterial community in such niches (Crump et al., 2007). A higher fraction of unknown bacterial genera observed in MWs suggests the potential of this unexplored ecosystem which might be crucial in understanding the MW channel dynamics in the light of enhanced glacial retreat.

The predominance of sequences affiliated to α-Proteobacteria, Flavobacteriia, Verrucomicrobia, γ-Proteobacteria and Acidimicrobiia in the Kongsfjorden waters is in accordance with the previous reports from Kongsfjorden (Jain and Krishnan, 2017; Sinha et al., 2017; Zeng et al., 2013). The relatively higher abundance of OTUs belonging to the genera *Sulfitobacter, Octadecabacter* and *Loktanella* (order *Rhodobacterales*, α-Proteobacteria) could be observed in the fjord water samples. Bacteria belonging to the genera *Rhodobacterales* are widespread and abundant in marine environments and their distribution and diversity in a Canadian Arctic marine system was elaborately evaluated by Fu et al. (2013). The most common *Roseobacter* clade belonging to *Sulfitobacter* and OTUs belonging to *Octadecabacter* as the primary surface colonizers and their role in community succession in the temperate coastal waters was reported by Dang et al. (2008). Members of *Sulfitobacter* and *Loktanella* were reported to be the predominant part of particle associated bacterial fraction in Kongsfjorden waters (Jain and Krishnan, 2017a) and are known to be associated with organic and inorganic marine particles (Slightom and Buchan 2009). Similarly, the dominance of OTUs belonging to *Pelagibacter ubique* belonging to the SAR11 clade of α-Proteobacteria in the outer KNS1 and KNBR stations is consistent with the results obtained by Sinha et al. (2017b). The ultramicrobial structure of *Pelagibacter* sp., which help them to escape from the protistan grazing and make them more competitive (Zhao et al., 2017) and their tolerance to wide range of salinity and temperature, make them the abundant class in marine habitats (Sinha et al., 2017b). The presence of the genera *Marimicrobium* belonging to the OM60 clade (γ-Proteobacteria) was observed within the top 10 OTUs in all the 4 fjord water samples and this clade is reported to be a marine representative (Yan et al., 2009). Similarly, the presence of OTUs belonging to the class Flavobacteriia were observed among the top 25 OTUs from the fjord stations (*Polaribacter, Fluviicola and uncultured Flavobacteriaceae*), the presence of which can be linked to their significant role in biogeochemical cycling of nutrients in the marine waters (Sinha et al., 2017b). Jain and Krishnan, 2017a have suggested the role of flavobacterial members such as *Polaribacter* in the degradation of phytoplankton derived dissolved substrates. The relatively higher abundance of OTUs affiliated to Verrucomicrobia observed in the inner fjord stations as compared to the outer station can be linked to the higher polysaccharide biomass associated with the inner fjord due to the brown algae/kelp ecosystem distributed along the coastal and inner zones (Buchholz and Wiencke 2016). The relatively higher abundance of OTUs belonging to the genera *Synechococcus* (Cyanobacteria) in the outer Kongsfjorden station as compared to the inner fjord stations, is an indication for the increased Atlantic water intrusion into the Kongsfjorden (Sinha et al., 2017b, Paulsen et al., 2016). The higher total organic carbon values obtained from the Kongsfjorden waters (21.7–31.1 ppm) indicates the enhanced organic matter inputs in Kongsfjorden which in turn could be a supporting evidence for the dominance of heterotrophic bacterial community known for complex organic matter degradation in the fjord. The dominant bacterioplankton community can have a great influence on the environment that they inhabit by contributing greatly to organic compounds cycling, such as the carbon cycle and it is been reported that approximately, 50% of the organic carbon is produced through heterotrophic bacterial metabolism (Ducklow 2000; Bunse and Pinhassi 2017). In the present study, the most dominant OTUs obtained belonged to genera *Octadecabacter, Pelagibacter*, OTUs belonging to *Flavobacteriaceae, Alteromonadaceae* etc which possess the ability to metabolize carbon. Thus, the overall bacterial community structure in the Kongsfjorden waters provided some insights into the functional role of fjord bacteria in organic matter degradation which is critical for keeping the ocean ecosystem functioning at healthy ratio.

Although next-generation sequencing technologies has opened a new dimension in unravelling the rare biosphere, most of the abundant taxa obtained from the metagenomic data are difficult to grow in the laboratory conditions which could limit our understanding of their physiological and functional potential in depth. Hence, culture based techniques stands significant in isolation and identification of bacterial strains and characterization of their physiology which can be correlated with the culture independent methods.

4.2. Culture dependent approach as a classical method to reveal the bacterial diversity

Diverse culturable bacteria were obtained from the glacier ice and MW samples and most of the isolated strains exhibited pigmentation which could be a possible UV protectant mechanism for their survival in the glacier environment (Lutz et al., 2017). It was observed from our study that dilute TSA media (1/10th strength) yielded the maximum CFU count in the first cultivation for the glacier ice and MW samples, which is in accordance with the previous works done by Antony et al. (2012) wherein they obtained higher retrieval yield on dilute TSA medium (1 and 10%) for the Antarctic ice core samples. The fact that oligotrophic bacteria cannot grow at higher substrate concentrations and cannot grow in nutrient-rich media upon first cultivation of organisms from natural habitats (Fry. 1990; Cho and Giovannoni. 2004) well explains our results. The inability to retrieve bacteria from the VB snow samples could be associated with their very low nutrient values as compared to the glacier ice, MWs and fjord waters (Table 1). For example, the nitrite values in VB snow was 15 times lower as compared to VB ice and 4 times lower as compared to the MWs while the nitrate values were 16 times lower than ice and

about 70 times lower than MWs. Similar trend was also observed for phosphate and silicate values of snow.

We could observe that among the VB ice isolates, except for the species *Paucibacter toxinivorans*, all others were previously reported from fresh water ecosystem, of which 50% of the isolates are reported from cold environment of glaciers. Similarly, about 46% of the strains isolated from the MWs have been previously reported from the cold habitats such as glacier ice, permafrost, tundra ecosystem, microbial mats, temporary ponds, sediments and glacier cryoconites (Supp Table S1) which testifies their adaptation to cold conditions. Among the VB ice isolates, *Polymorphobacter glacialis* was found to be the dominant species followed by species representing *Flavobacterium, Herminiimonas* and *Cryobacterium*. The first report of *Polymorphobacter glacialis* is from the ice core of Muztag glacier, China (Supp Table S1). The dominance of genus Flavobacterium from both Arctic and Antarctic ice packs by cultivation was reported Brinkmeyer et al. (2003) while there are reports on the presence of *Herminiimonas* spp. from Antarctic glacier (García-Echauri et al., 2011) and from Greenland sea-ice brine (Møller et al., 2011). The Actinobacterial genera *Cryobacterium* have been reported as one of the dominant bacterial taxa from the cryoconite hole samples from Broggerbreen glaciers, from Ny-Alesund (Singh et al., 2014) and were reported for their cold active enzyme activities. Majority of the isolated strains from the MWs belonged to *Pseudomonas* spp. which are previously reported from the Arctic region and known for their cold-active enzyme production (Vardhan Reddy et al., 2009). Production of cold active enzymes can help the bacteria to thrive under glacier-cold conditions and to utilize the available organic matter in the oligotrophic environments of glacier and proglacial channels (Singh et al., 2016). The isolates belonging to the order *Burkholderiales* were previously reported for their metabolic diversity and niche-specific functions such as cyanobacterial hepatotoxin degradation, hydrogen oxidation, and pigment production (Supp Table S1). However, the isolates obtained in this study have not been tested for this phenotype. Few bacterial genera like, *Polaromonas, Sphingomonas* and *Flavobacterium* which were identified through amplicon sequencing based approach were also observed to be a dominant fraction in the culture based study. Among the bacterial isolates retrieved from the melt waters, a minor fraction were observed to be closely affiliated to bacteria previously reported from Arctic tundra (*Pseudarthrobacter oxydans*) tolerant to toxic metal such as mercury (Balan et al., 2018) and from Tibetan permafrost (*Pseudarthrobacter sulfonivorans)* known for degradation of crude oil and multi benzene compounds (Zhang et al., 2016) (Supp Table S1). These observations suggest the highly flexible nature of the MW isolates to withstand various environmental stresses and hence a detailed genome based study on such candidates is pre-requisite to understand how they have evolved such resistant mechanisms in the cold polar environment. The cultivable fraction of bacterial communities from the glacier ice and MWs also revealed the presence of potential novel bacterial isolates (with <98.7% similarity with closest phylogenetic neighbors) which is in accordance with the previous studies that showed the presence of rare taxa in glacially fed streams (Wilhelm et al., 2014). These rare communities might be lost with the ongoing retreat of glacial snow and ice driven by climate change, therefore systematic studies of such glacial ecosystem is critical.

For the Kongfjorden water samples, maximum retrievable counts were obtained from Zobell Marine Agar (1/4th strength) (Supp Table S4). Previous studies have reported the use of ZMA media for the enumeration of viable heterotrophic bacteria from marine environment of Kongsfjorden (Sinha et al., 2017a; Prasad et al., 2014; Srinivas et al., 2009). Among the isolates retrieved from Kongsfjorden waters, 63% were previously reported to be of marine origin while 31% were of fresh water origin and about 56% of the total were found to be isolated from cold environments/Polar regions (Supp Table S1). The Gammaproteobacterial members belonging to the genera *Alteromonas, Pseudoalteromonas, Pseudomonas* and *Psychrobacter* retrieved in our study were previously reported for their extracellular enzyme production and role in degradation of dissolved organic matter and carbohydrates in the cold marine environment of Kongsfjorden (Jain and Krishnan, 2017b). Among the α-proteobacterial candidates, the dominance of phylotype affiliated with the genus *Loktanella* could be observed, which were previously reported from Kongsfjorden waters (Jain and Krishnan, 2017; Sinha et al., 2017) as well as from the interface between the ocean and a fresh water spring (Park et al., 2013).

While comparing the VB ice, MWs and fjord system, it was observed that majority of the culturable isolates identified from VB ice and MWs were retrieved from 4 °C incubations while the isolates identified from Kongsfjorden waters were more from 20 °C. This could explain the differences in the culturable bacterial community between the systems, wherein the glacier associated system with its cold temperatures supports more of the cold loving bacterial community while the fjord system with comparatively warmer waters supports bacteria which could grow better at higher temperature incubations (20 °C).

The detection of isolates/OTUs belonging to the genus *Flavobacterium* from all three sampling sites in both culture based and high throughput sequencing approach, strongly indicates the adaptability of this genus to cope up with salinity and temperature fluctuations associated with the fresh water system of glacier and MWs as well as the marine fjord system. Similar conclusions can be drawn for the presence of *Salinibacterium* sp. in both MWs as well as fjord system. This might also suggests that such species have the potential to thrive in oligotrophic conditions which prevail in the glaciers and MWs as well as the high organic rich conditions that exist in the fjord waters. Comparison of the culturable data with the high-throughput sequencing data revealed, out of the 42 genera retrieved in the cultivable approach, 25 were detected in amplicon sequencing based approach, while 17 genera were represented only by the culturable isolates (Supp data Table S1). Similar observations were reported by Chen et al. (2016) and Sinha et al. (2019), wherein in the former, the authors while comparing the actinobacterial genera using culture dependent and pyrosequencing approach, observed that 14 actinobacterial genera in the cultivable approach were not detected in the pyrosequencing data. In the later paper, the authors while comparing the bacterial community structure using culture based and culture independent (16S rRNA amplicon sequencing) approach, have obtained the retrieval of 5 bacterial genera in the cultivable approach which was not detected in the high throughput sequencing data. These results reveal the importance of culture based approach in bacterial diversity studies associated with the rare biosphere of Arctic. The potential novel bacterial isolates obtained in our culture based study also suggest the significance of culturing bacteria as a tool to unravel the unknown taxa and for understanding their functional roles in the ecosystem. Therefore a combination of NGS based and culture based approach is prerequisite in obtaining a comprehensive picture on bacterial diversity.

4.3. Differences in the bacterial community structure between the sampling sites and their correlation with environmental parameters

The glacier snow, MWs and fjord waters exhibited significant difference in their bacterial community composition as per the cluster dendrogram and heatmap plot (Fig. 4). The top abundant OTUs that were repeatedly encountered in the NGS analysis of MW samples were also found to be dominant in the VB ice. The venn diagrams plotted could also explain the same in detail. From

Fig. 5(c), it is evident that VB ice shared more number of OTUs with the MWs as compared to the fjord waters. The comparable nutrients, ions, total organic carbon and trace element values for glacier ice and MWs also suggests the similar nature of these systems which supports the presence and abundance of same bacterial OTUs. Since environmental conditions are known to shape bacterial community compositions, the presence of shared OTUs in similar environmental conditions may not be the only reason that can conclusively indicate sourcing. Further quantitative analyses of the data in this regard is expected to give better clarity on the same.

A significant contribution of the OTU 1,105,280 and OTU 331 belonging to *Burkholderiaceae* and the OTU 101,660 and OTU 520 belonging to *Rhodobacteraceae* to the overall % dissimilarity between the samples was observed in the SIMPER analysis. The high proportion of sequences belonging to *Rhodobacteraceae* in the fjord samples could be linked with their particle associated nature enabling them to associate with organic and inorganic marine particles/surfaces in the Kongsfjorden (Jain and Krishnan, 2017a) while the dominance of OTUs belonging to *Burkholderiaceae* in the VB glacier snow highlights their resistance to cold oligotrophic conditions of glaciers and their free-living nature (Coenye and Vandamme, 2003).

Analysis of linkages between the bacterial community structure and the various significant environmental parameters using distance based-RDA yielded a strong correlation of nutrients (NO_3^- and SiO_4^{2-}) with glacier ice as well as the MW bacterial community. Glaciers in Svalbard, receive modest rates of nutrient deposition that may be highly variable from year to year due to extreme episodic events (Hodson et al., 2005). The supraglacial environments promote ammonium ion assimilation and nitrification within the cryoconite holes while subglacial environment appears to promote denitrification due to the prevalent anoxic conditions (Hodson et al., 2005). The geochemical data from the Austrebroggerbreen glacier sites suggests that the Broggerbreen glacier forefields were geochemically significant in the context of silicate weathering (Hodson et al., 2005). This could very well explain the comparatively higher nutrient values in the glacier and MW channels as compared to the fjord waters, which in turn might have influenced the distinct bacterial community structuring of these ecosystems. The lower nutrient values in fjord waters was comparable with the previous reported values of nutrients from the Kongsfjorden waters (Bazzano et al., 2014) and indicated photosynthetic activities to be the major cause for nutrient depletion during summer. Temperature exhibited positive correlation with Kongsfjorden waters, which can be related to the Atlantic water intrusion to the fjord which in turn is a crucial factor determining the fjord properties (Sinha et al., 2017a). Trace metals, Cl^-, and SO_4^{2-} ions were found to be linked with the fjord bacterial community as per the db-RDA plot. The bacterial community in the Kongsfjorden are mostly marine in origin which are capable of tolerating high salt concentration and degrading organic matter (as indicated by the high TOC values in the fjord). Anions such as Cl^-, and SO_4^{2-} ions are essential for marine bacteria to cope with the salt stress by acting as osmolytes as well as essential for their growth (Roeßler et al., 2003) while trace elements such as Fe, Mn, Zn, Cu and Ni play significant role in microbial cycling of major nutrients like C and N in oceans (Morel and Price 2003). Sinha et al., (2017a) have suggested that the phytoplankton composition and concentration along with the mild temperatures in the fjord are the major determining factors for the Kongsfjorden bacterial diversity. Thus, it can be inferred that the Kongsfjorden bacterial community varies with changes in the physico-chemical properties of the fjord which in turn is majorly influenced by inflow of warm Atlantic water and is significantly different from the valley glacier and MW bacterial community.

5. Conclusion

The significant variation in the bacterial community composition of the Vestrebroggerbreen valley glacier, its associated melt water channels and the downstream marine system of Kongsfjorden ($R = 0.873$, $p = 0.001$) suggests the influence of environmental variables and dispersal vectors in determining the patterns of bacterial diversity. The VB snow exhibited the lowest diversity with unknown genera belonging to *Burkholderiaceae* dominating the community while we could observe a highly diverse (Shannon index between 6.26 and 10.67) bacterial community in the MWs wherein the dominant bacterial genera were *Flavobacterium*, *Leptolyngbya*, *Polaromonas*, *Methylotenera* and *Bdellovibrio.* The presence of many unknown taxa in the MWs in both culture based and high throughput sequencing study, highlights the importance of such transient niches as conducive hotspots for unknown microbiota. The presence of shared OTUs along with comparable nutrients, ions, total organic carbon and trace element values for glacier ice and MWs suggests the similar nature of these systems which in turn supports the presence and abundance of similar bacterial community, while the downstream Kongsfjorden bacterial community was dominated by heterotrophic taxa known for the degradation of organic matter as well as those known to be indicators of Atlantic water intrusion. Our results suggests the added value of examining bacterial communities to better understand and trace the transport of meltwaters from their glacial source to the oceans, since we could observe a gradual loss in the glacier associated taxa on reaching the fjord system. However, further quantitative analyses are needed to better understand sourcing of bacterial communities to the downstream ecosystem.

Authors contribution

Conceptualization: K.P.Krishnan, Femi Anna Thomas; Data curation: Femi Anna Thomas, Rupesh Kumar Sinha; Formal analysis: Femi Anna Thomas, Rupesh Kumar Sinha; Funding acquisition: K.P.Krishnan; Investigation: Femi Anna Thomas; Methodology: K.P. Krishnan, Femi Anna Thomas, Rupesh Kumar Sinha; Project administration: K.P.Krishnan; Supervision: K.P.Krishnan; Writing – original draft: Femi Anna Thomas; Writing – review & editing: K.P. Krishnan.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors wish to express their gratitude to Director, ESSO-NCPOR for his interest in this work. Thanks are due to Dr. Anand Jain, Dr. Lavkush Patel and Ms. Archana Singh, Scientists at ESSO-NCPOR for their help in sampling. We acknowledge Dr. Mahesh Mohan, Mr V. G. Gopikrishna, Mr. V. M. Kannan, Mr. John Richard Thomas from Mahatma Gandhi University, Kerala and Ms. Melena Soares and Dr. P.V. Bhaskar, Scientists at ESSO- NCPOR for their timely help with the trace metal, ion chromatography and Total organic carbon analysis. Thanks are also due to Mr. Rahul Dey, Research Fellow at ESSO-NCPOR for his help with the R platform. We are grateful to Dr. Siddarthan Venkatachalam, Scientist at ESSO-NCPOR for critically reviewing the manuscript for content and language. This work was undertaken as part of the project 'Study of physiology of cold adapted bacteria collected from ice

and snow from the Poles'. This is ESSO-NCPOR contribution number **J-42/2019-20**.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.135264.

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Antonie van Leeuwenhoek
https://doi.org/10.1007/s10482-021-01604-9

ORIGINAL PAPER

Bacterial diversity and their metabolic profiles in the sedimentary environments of Ny-Ålesund, Arctic

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Received: 25 November 2020 / Accepted: 1 June 2021
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Abstract Sedimentary environments in the Arctic are known to harbor diverse microbial communities playing a crucial role in the remineralization of organic matter and associated biogeochemical cycles. In this study, we used a combination of culture-dependent and culture-independent approaches to understanding the bacterial community composition associated with the sediments of a terrestrial versus fjord system in the Svalbard Arctic. Community-level metabolic profiling and growth response of retrieved bacterial isolates towards different carbon substrates at varying temperatures were also studied to assess the metabolic response of communities and isolates in the system. Bacterial species belonging to *Cryobacterium* and *Psychrobacter* dominated the terrestrial and fjord sediment retrievable fraction. Amplicon sequencing analysis revealed higher bacterial diversity in the terrestrial sediments (Shannon index; 8.135 and 7.935) as compared to the fjord sediments (4.5–5.37). Phylum Proteobacteria and Bacteroidetes dominated both terrestrial and fjord sediments. Phylum Verrucomicrobia and Cyanobacteria were abundant in terrestrial sediments while Epsilonbacteraeota and Fusobacteriia dominated the fjord sediments. Significant differences were observed in the carbon substrate utilization profiles between the terrestrial and fjord sediments at both 4 °C and 20 °C incubations ($p < 0.005$). Utilization of N-acetyl-D-glucosamine, D-mannitol and Tween-80 by the sediment communities and bacterial isolates from both systems, irrespective of their temperature incubations implies the affinity of bacteria for such substrates as energy sources and for their survival in cold environments. Our results suggest the ability of sediment bacterial communities to adjust their substrate utilization profiles according to condition changes in the ecosystems and are found to be less influenced by their phylogenetic relatedness.

Keywords Culture-dependent · Terrestrial · Fjord · Community-level metabolic profiling · Growth response · Amplicon sequencing

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10482-021-01604-9.

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Published online: 20 June 2021

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Introduction

The Arctic is considered as a 'global hotspot' that is highly sensitive to climate change (IPCC 2013). Reduction in Arctic sea ice, glacier ice and snow, thawing of permafrost, and increase in productivity of the vegetation are some of the most direct impact of globally rising temperatures on Arctic ecosystems (Descamps et al. 2017). The Svalbard archipelago is one of the regions which is experiencing the fastest temperature increase within the circumpolar Arctic, along with the highest rate of sea ice loss and glacier retreat over the past three decades (Nuth et al. 2013).

The tidal and land terminating glaciers in Svalbard deliver sediment-laden freshwater into the downstream fjord systems, which in turn show strong physico-chemical as well as biological gradients along the head-to-mouth (glacier-to-ocean-influenced) axis. Our previous study on a glacio-marine transect in Svalbard Arctic reported significant differences in the bacterial community structure associated with a land terminating glacier (ice and snow), its pro-glacier melt waters and downstream fjord waters indicating the unique nature of these systems and the influence of environmental variables in determining the patterns of bacterial diversity (Thomas et al. 2020). The sediments associated with such glacier influenced and fjord environments also represents complex microbial habitats supporting distinct bacterial and archaeal communities with complex metabolism (Stibal et al. 2012; Anesio et al. 2017; Zeng et al. 2017; Teske et al. 2011). These microbial fraction comprise the bulk of the biomass and are crucial in remineralization of organic matter and associated biogeochemical cycles (Stibal et al. 2012; Lin et al. 2017). So far, there are numerous reports on microbial diversity, physiology and functions associated with supraglacial cryoconite sediments (Edwards et al. 2011; Lutz et al. 2017; Poniecka et al. 2020), subglacial systems (Skidmore et al. 2005; Stibal et al. 2012; Sułowicz et al. 2020) and glacier forefield soils (Kim et al. 2017; Nash et al. 2018; Malard et al. 2019), but studies on glacier snout and forefield associated sediments are limited to a single culture-dependent study from the sediments of a meltwater stream from Midtre Lovénbreen glacier (Reddy et al. 2009). They have reported the cold-active enzyme production and pigmentation properties of pro-glacier sediment bacteria and indicated the presence of Proteobacteria, Cytophaga-Flavobacterium-Bacteroidetes and high G + C Gram-positive bacteria as common inhabitants of these habitats. Since the glacier snout and forefield sediments are relatively unexplored in terms of microbial studies, it would be interesting to understand their bacterial community structure and how their metabolic signature reflects the glacier environment.

However, there are many studies highlighting the microbial community structure (Zeng et al. 2017; Conte et al. 2018; Fang et al. 2019) and functions (Teske et al. 2011; Buongiorno et al. 2019) associated with Svalbard fjord sediments. To highlight a few, Fang et al. (2019) have observed significant differences in the bacterial community structure between sediments of inner, middle and outer fjord basins and reported the influence of location depth, temperature and salinity on sediment community. The increased influx of Atlantic waters into the Kongsfjorden system influencing sediment community composition from the outer to the inner fjord has been reported by Zeng et al. (2017). Teske et al. (2011) reported higher rates of hydrolytic activities for Kongsfjorden sediment bacterial communities to hydrolyze polysaccharides and algal extracts as compared to the seawater communities. They also reported the presence of some microbial key populations (Bacteroidetes) having specific enzymatic capabilities, for example to readily hydrolyze substrates which were found to be recalcitrant in surface and bottom waters and suggested the need to test the linkages between enzymatic potential and phylogenetic identity using cultured isolates.

These studies are indicative that the bacterial communities associated with the Arctic glacio-marine sediments tend to exhibit distinct physiological properties and metabolic functions driven by the substrate availability and the various environmental conditions of each system. Therefore, assessing the metabolic potential of microbial communities along with their community structure is crucial in understanding their ecological role in such complex environments. The cultivable bacterial fraction, although accounting for as little as 1% of the total bacterial community, provides opportunities in exploring the metabolic diversity and traits within microbes inhabiting these environments.

Furthermore, in the light of enhanced warming scenario in the Arctic, with observed and projected annual average Arctic warming approximately twice the global mean (Overland et al. 2019), it is also

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imperative to understand the influence of temperature on the metabolic response of the total community as well as on different bacterial species of the terrestrial and fjord system. Kritzberg et al. (2010) studied the bacterial response in terms of production and respiration from the Fram strait waters by experimental manipulations of temperature and resources in combinations. They reported enhanced bacterial production and respiration following a temperature increase of + 6 °C from the in-situ conditions and the response to temperature was higher in resource amended treatments, indicative of a substrate-temperature interaction regulating the bacterial metabolism.

Keeping these aspects in mind, the following questions were posed 1) whether the total sediment community metabolic response towards ecologically important substrates in the terrestrial and fjord systems are different from the metabolic response exhibited by individual bacteria isolated from such systems (2) whether phylogenetically-related species tend to exhibit similar metabolic responses or whether sediment-type and origin influence their metabolic responses and (3) whether temperature variations have an influence on the metabolic profiles of the total community as well as on different bacterial species of the terrestrial and fjord system. In order to address these questions, in the present study we aim to explore the taxonomic diversity and metabolic potential of the total community as well as the retrievable fraction of a terrestrial versus fjord sediment system in the Svalbard. Cultivation-dependent and cultivation-independent high throughput amplicon sequencing approach was followed accompanied with measurement of physico-chemical parameters associated with each system. Community-level physiological profiling (CLPP) of the sediments using EcoPlates^{TM} and growth response of retrievable bacteria towards different carbon substrates, with varying temperatures were studied. Such studies would help in estimating and eventually predicting the bacterial responses to varying substrate availability in the highly changeable Arctic ecosystems.

Materials and methods

Study area and sampling strategy

The study area covered Vestrebroggerbreen (VB) glacier which is a land terminating polythermal glacier in Ny-Ålesund (4.7 km^2 long) and the downstream fjord system–Kongsfjorden (Fig. 1). The meltwater channels starting from the glacier snout drains through the Broggerbreen valley and finally discharges into the Kongsfjorden. The Kongsfjorden is a glacio-marine influenced fjord system located between 78° 04′ N–79° 05′ N and 11° 03′ E–13° 03′ E (Fig. 1). Kongsfjorden is influenced by Atlantic and Arctic water masses as well as by the glacial influx of sediment-laden freshwaters which in turn affect the ecosystem dynamics by creating strong environmental gradients and changes in community structure (Hop et al. 2002).

A total of 3 sediment samples were collected from the Vestrebroggerbreen glacier snout region (VB1–VB3) and pooled together to form a single representative sample (VB). Similarly, 3 sediment samples were collected from the glacier foreland region (BR1–BR3), and a homogenous single sample was obtained as BR (Fig. 1). Surface sediment samples were collected across Kongsfjorden from the point where the meltwater channels empty into the fjord (KNBR), from the outer fjord mouth (KNS1), and the inner fjord (KNS9) region (Fig. 1) using Van-veen grab deployed from *MS Teisten* (https://nyalesundresearch.no/infrastructures/ms-teisten/). VB and BR sediments are representatives of the Arctic terrestrial system while KNBR, KNS1, and KNS9 sediments represent the fjord system. The overlying water temperature and salinity were measured using a Waterproof Portable meter (Cyberscan series 600) (Eutech instruments, Thermo Fisher Scientific, USA) for all the sampling locations. All the samples were collected in sterile sampling bags (Nasco Whirlpaks, Himedia, India) and were immediately transferred to − 20 °C until further processing. An aliquot of the samples was maintained at 4 °C for cultivation-dependent analysis. The samples were then transported in a frozen state to the home laboratory at National Centre for Polar and Ocean Research, India for analysis.

Chemical analysis of sediment samples

The pH of the sediment pore water was measured by centrifuging the sediment sample and measuring the pH of the supernatant water using Cyberscan pH meter 510 (Eutech Instruments, Thermo Fisher Scientific, USA). Water content (5 g of each sample) was measured as gravimetric weight loss after drying the sediment at 105 °C until constant weight. Organic and

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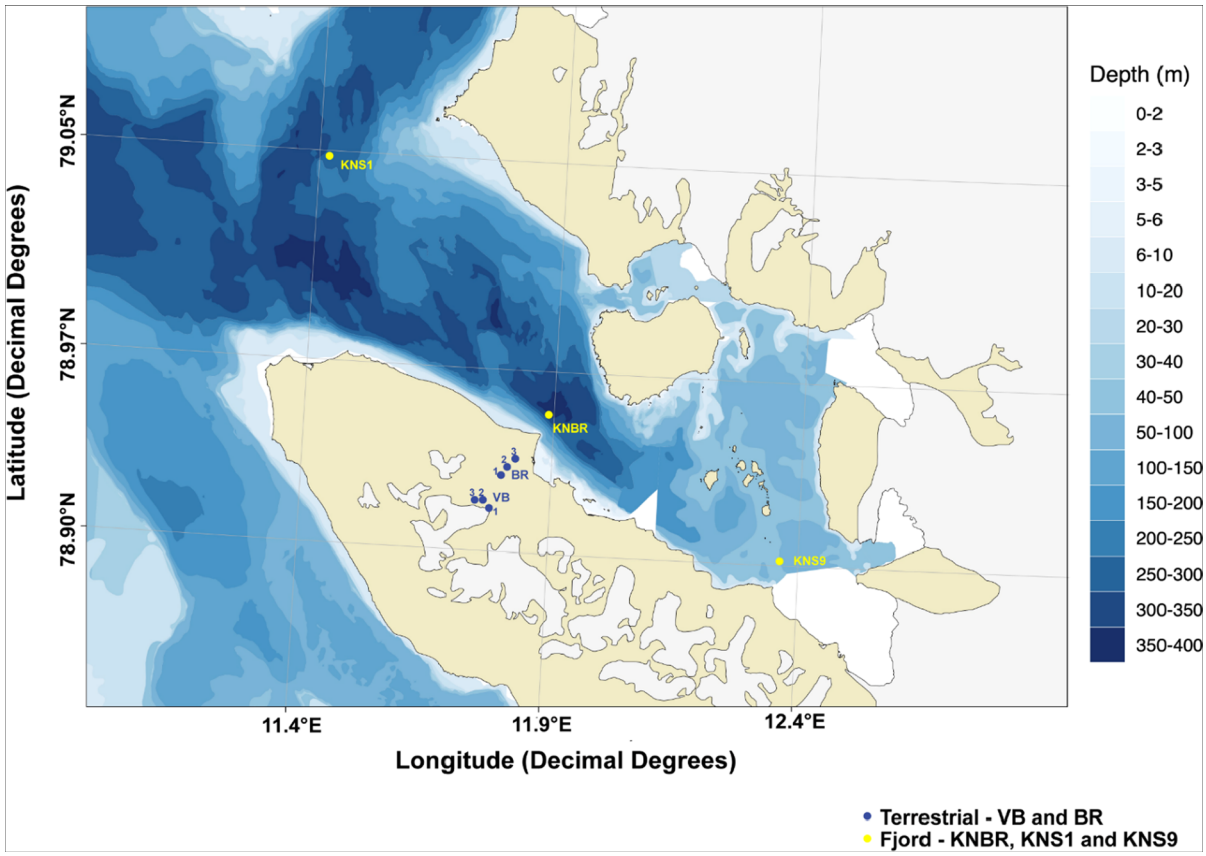

Fig. 1 Map of the glacierized terrestrial regions and Kongsfjorden with the sediment sampling points (Vestrebroggerbreen (VB) glacier snout, foreland (BR) and fjord (KNBR, KNS1 and KNS9)) marked (https://github.com/MikkoVihtakari/PlotSvalbard)

inorganic carbon were analyzed as per Winkelmann and Knies (2005) using PRIMACS^{MCS} TOC analyser (SKALAR, Netherlands). Sediment samples were analyzed for trace elements using an Inductively-coupled Plasma Mass Spectrometer (Thermo iCAP Q ICP-MS, Thermo Fisher Scientific, USA). Sample preparation involved microwave digestion as detailed by Lu et al. (2013) and the USEPA method 3051, followed by a final dilution of 25 times with MilliQ. Total Mercury was analyzed using Direct Mercury Analyser (Duel cell-DMA 80, Milestone, Italy).

Total microbial abundance

Total sediment microbial count was carried out according to Epstein et al. (1997) using 4, 6-di-amidino-2-phenylindole (DAPI) with minor modification. In brief, 0.5 g of sediment in 50 mL of filter-sterilized saline (0.9%) was vortexed for 10 min followed by ultra-probe sonication (at 40% speed over 80 s with interruption of 2–4 times) using an Ultrasonic homogenizer (Model 3000, Biologics, Inc, USA) for dislodging cells from sediment particles. A 2 mL aliquot of the sample was then incubated with DAPI (20 µL of 1 µg mL^{-1} working solution per mL) for 5 min and filtered through black 0.22 µm membrane filters (Nucleopore Track-Etch Membrane, Whatman). The total count was enumerated under the Leica DM6 B microscope (Leica Microsystems, Wetzlar, Germany).

Isolation and identification of retrievable heterotrophic bacteria

Retrievable bacterial fractions from the terrestrial and fjord sediments were enumerated by the spread plate method (Sanders 2012). The sample suspension was prepared by sonicating 5 g of sediment in 45 mL of

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filter-sterilized saline (0.9%). After serial dilutions (10^{-2} and 10^{-4} dilutions), the sediment suspension (100 µL) was plated onto solid R2A media (1/2 strength), Antarctic Bacterial Medium (ABM), Tryptone Soy Agar (TSA) (1/10th strength), Actinomycete isolation agar (AIA) and Zobell Marine Agar (ZMA) medium (1/4th strength). The plates were then incubated at 4 °C and 20 °C for 2–3 weeks and bacterial isolation and purification was carried out based on unique morphological characteristics.

The bacterial genomic DNA was extracted from the pure bacterial cultures using ChargeSwitch gDNA mini bacteria kit (Invitrogen, USA). The extracted DNA was subjected to Rep-PCR amplification with the BOX A1R primer (Yang and Yen 2012) and based on their unique banding patterns, isolates were chosen for 16SrRNA gene amplification (Sinha et al. 2017) followed by sequencing using 3500 Genetic Analyzer (Applied Biosystems, Thermofisher Scientific, MA, USA). The obtained sequences were trimmed and assembled using Codon code Aligner software Version 8.0.2 and the consensus contigs were chimera checked using Pintail 1.1 software. The 16S rRNA gene nucleotide sequences were analyzed on EzBioCloud server against validly published type strains of bacterial species (Yoon et al. 2017). The identified 16S rRNA gene sequences (104 sequences) were submitted in GenBank under the accession numbers MN080149–MN080222, MT309496–MT309525.

Bacterial community analysis using amplicon sequencing and downstream bioinformatic processing

Environmental DNA from the sediment samples (0.5 g of sediment) was extracted using DNeasy Power Soil kit (Qiagen, USA). The DNA concentration and purity were measured using the Qubit fluorometer (Thermo Fisher Scientific, USA) and by gel electrophoresis on 1% agarose. The DNA samples were sequenced on the HiSeq platform (Illumina, USA) using Pro341F/Pro805R primers for V3–V4 hypervariable regions of the 16S rRNA gene (Takahashi et al. 2014). The raw nucleotide sequences were deposited in the NCBI Sequence Read Archive (SRA) database under the SRA accession number: PRJNA475645. The downstream processing of raw DNA sequences was carried out according to the protocols detailed by Bolyen et al. (2019) using QIIME2 software. The demultiplexed raw sequence datasets were trimmed for the removal of 16S rRNA gene primers and multiplexing adapters by using QIIME2 Cutadapt plugin. Further, the sequences were subjected to denoising, dereplication, and chimera filtering using the DADA2 denoise-paired method of QIIME2. The clustering of reads into features was performed against SILVA_132 marker gene reference database (Quast et al. 2013) by training the database using the q2-feature-classifier plugin. Unique representative features/OTUs from each sample were further subjected to taxonomic classification using the classify-consensus-vsearch method in q2-feature-classifier plugin with 0.99% identity threshold. This was followed by taxonomy-based filtering to remove the sequences representing chloroplasts and mitochondria using the q2-taxa plugin and rarefaction of the OTU counts (rarefaction depth–9458, 10 iterations) before the downstream diversity analysis. The alpha diversity estimation was carried out using the q2-diversity plugin.

Community-level physiological profiling using EcoPlate

Biolog EcoPlate™ (Biolog Inc., USA) was used to evaluate community-level metabolic responses. The EcoPlate™ contains three replicate wells of 31 carbon substrates (Garland and Mills 1991) and control well (A1) with no added carbon substrate. The 31 substrates used in the EcoPlate™ represented carbohydrates, polymers, carboxylic acids, and amino acids, amines and amides. Carbon substrate utilization by microbes is indicated by the respiration-dependent reduction of the tetrazolium dye leading to a purple coloration which was quantified spectrophotometrically using a microplate reader (Synergy 2-multi mode microplate reader, BioTek Instruments, USA). Preparation of sediment samples for EcoPlate™ analysis was performed as detailed by Sinha et al. (2019) and the EcoPlates were incubated at both 4 °C and 20 °C to understand the influence of temperature on the metabolic potential of the sediment community. The intensity of color development was measured at a wavelength of 595 nm for 240 h (24 h time interval). The most intensive metabolism of carbon substrates was observed at 192 h for 4 °C and 216 h for 20 °C incubation. The results were expressed as Average Well Color Development (AWCD) and the Shannon–

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Wiener (H) and Richness (Rs) indices were computed. The AWCD was evaluated according to Garland and Mills (1991) following formula AWCD = $\sum(OD_i)/31$; where $OD_i = C - R$. C is the optical density measurement within each well and R is the absorbance value of the control well. The primary data were further normalized following the equation $(C - R)/AWCD$ separately for each substrates using respective AWCD values. For the percentage utilization of each substrate category, the normalized values for each category were divided by the $\sum(C - R)/AWCD$ values. The Shannon–Wiener (H) index was calculated using the formula $H = -\sum pi(lnpi)$; where pi is the ratio of the activity on each substrate (OD_i) to the sum of activities on all substrates $\sum OD_i$ (Gomez et al. 2006). Richness index (R) is the number of substrates metabolized by the microbes in each sample.

Understanding the growth yields of selected retrieved isolates of terrestrial and marine origin towards different carbon substrates

While the total sediment community metabolic profile was obtained from Ecoplate assay, it is imperative to understand how individual retrieved bacteria from terrestrial and marine samples could respond to different ecologically important substrates. For this purpose, from a total of 27 different species retrieved from the terrestrial sediments and 15 different species retrieved from the fjord sediments, we chose a total of 20 different species−10 belonging to the terrestrial sediments and 10 from the fjord sediments. Isolates retrieved from both 4 and 20 °C incubation were chosen based on their relative abundance (List of isolates given in Table 5). The fully grown bacterial cultures in the broth media were centrifuged and the resulting pellet was washed multiple times with 0.9% saline. The bacterial inoculum thus obtained was fixed to an optical density (OD) of 0.2 at 600 nm and the corresponding number of bacterial cells was measured using DAPI staining (protocol same as total microbial abundance). The substrate utilization experiment was carried out in microplates wherein 10 µL culture aliquot (with fixed OD of 0.2) was added to each well which was preloaded with 140 µL of a substrate minimum medium mix. Substrates tested included carbohydrates (D-cellobiose, sucrose, D-mannitol, erythritol, D-lactose, D-glucose, N-acetyl-D-glucosamine, and D-xylose), polymers (Tween 80, cellulose), amino-acids (L-phenyl alanine, L-serine, DL-Asparagine, L-glutamic acid), and carboxylic acids (D-malonic acid, D-galacturonic acid, p-aminobenzoic acid, DL-malic acid, and sodium pyruvate). Substrates were chosen based on the Biolog EcoPlate™ substrate list with few additional substrates included and were filter-sterilized before use. Each substrate was added to a Minimal media (MM, composition as described by Freese et al. (2010) and Pfennig (1974)) to produce a final concentration of 50 mg/L (Freese et al. 2010). The experiment was carried out in triplicates with controls comprising of MM with bacteria and without substrates, MM with substrates and without bacteria, and MM without bacteria and substrates. The experiment was tested at both 4 and 20 °C temperature incubations to check the effect of temperature on bacterial growth by the utilization of different carbon substrates. Absorbance (OD 600 nm) was measured for 15 days at regular intervals of 0, 3, 6, 9, 12, and 15 days.

For each of the selected bacterial isolates, the optimum temperature and temperature range for growth was determined on diluted R2A broth media (50% w/v of the original strength of 3.12 g/L) for terrestrial isolates and diluted ZMB (25% w/v of the original strength of 40.25 g/L) for fjord isolates at different temperature incubations (4, 10, 15, 20, 25, 30, and 35 °C) by measuring optical density at 600 nm.

Statistical analysis

The influence of geochemical properties in explaining the bacterial community structure associated with the terrestrial and fjord sites was done by Canonical Correspondence Analysis (using Bray Curtis dissimilarity) using CANOCO 4.56 statistical software. Pearson linear correlation was calculated using OriginPro 9.0 software to measure the correlation between geochemical properties and total bacterial community structure, bacterial community and Ecoplate substrate utilization as well as between geochemical properties and Ecoplate substrate utilization. Principal component analysis (PCA) was performed using the Canoco 4.56 package to ordinate the strains depending on the carbon sources they were able to metabolize. One-way Analysis of Variance (ANOVA) was performed for examining significant differences between the metabolic profiles of terrestrial and fjord sediment

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communities as well as between the retrievable bacterial isolates of the two systems, at 4 °C and 20 °C incubations.

Results and discussion

Geochemical properties of sediment samples

The overlying water temperature for the terrestrial sediments varied between 0.9 and 1.3 °C and salinity was 0.08 PSU, while that of the fjord sediments varied between 3.8 and 5.9 °C and 34.3–34.9 PSU (Table 1). The highest value of sediment pore water pH was observed in VB and BR sediments (~ 8.4) while the pH of the fjord sediment pore water ranged from 7.9 to 8.1 (Table 1). The total organic carbon (TOC) content among the terrestrial and fjord sediments varied between 1.2 and 2.9% with the highest value recorded in the outer fjord sediment (KNS1) and the lowest value noted in the foreland sediment (BR). A reduction in the concentration of TOC was observed from the outer to the inner fjord sediments while an increase in Inorganic carbon (IC) concentration was noted towards the inner fjord sediments (Table 1). The highest TOC values observed in the outer fjord sediment with a reduction noted from outer to the inner fjord sediments corresponds to the previous reports (Lu et al. 2013; Kumar et al. 2016). High turbidity towards the fjord head (inner fjord) and steep gradient in sedimentation rate along with the supply of terrigenous carbonates and organic matter deprived glacier materials towards the fjord head may be critical in producing such a clear spatial gradient in TOC and IC values in the fjord sediments (Kumar et al. 2016; Koziorowska et al. 2017). The highest values of Mn (362.0 mg/Kg), Co (13.2 mg/Kg), Cu (33.6 mg/Kg), Zn (223.9 mg/Kg), Ni (40.8 mg/Kg) and Cd (0.3 mg/Kg) were found in terrestrial sediments. Similarly, the outer fjord sediment, KNS1 exhibited the highest concentrations of Pb (7.9 mg/Kg) and Hg (90.9 µg/Kg) (Table 1). The higher

Table 1 Geochemical properties, total microbial counts (TMC) and retrievable heterotrophic bacterial counts (RHBC) in the sediments

Parameters	Glacier sediments		Fjord sediments		
	VB	BR	KNBR (depth-126 m)	KNS1 (depth-240 m)	KNS9 (depth-46.5 m)
Temperature (°C) of overlying water	0.9	1.3	4.7	3.8	5.9
Salinity of overlying water (PSU)	0.08	0.08	34.9	34.9	34.3
Sediment pore water pH	8.4	8.4	7.9	8.0	8.1
Water content (%)	20.4	15.4	18.9	20.5	20.9
TOC (%)	1.9	1.2	2.3	2.9	1.3
IC (%)	6.3	1.3	2.8	2.2	3.1
Mn (mg/Kg)	205.3	362.0	250.5	314.1	332.6
Ni (mg/Kg)	40.8	16.1	20.8	21.3	26.6
Co (mg/Kg)	8.3	13.2	9.4	11.7	11.9
Cu (mg/Kg)	12.6	33.6	19.1	21.2	19.1
Zn (mg/Kg)	166.7	223.9	152.9	136.1	117.3
Cd (mg/Kg)	0.3	0.1	0.2	0.2	0.1
Pb (mg/Kg)	3.9	2.9	3.0	7.9	2.6
Hg (µg/Kg)	44.8	18.2	38.6	90.9	22.1
TMC (cells/g)	6.2×10^6	4.3×10^6	2.2×10^8	4.9×10^8	4.7×10^8
RHBC at4 °C (CFU/g)	9.1×10^3*	3.2×10^3*	1.6×10^5**	2.5×10^5**	2.5×10^5**
RHBC at 20 °C (CFU/g)	2.8×10^3*	1.1×10^3*	7.7×10^4**	1.1×10^5**	8.5×10^4**

*On half-strength R2A medium

**On quarter-strength ZMA

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Table 2 List of retrievable heterotrophic bacterial isolates from the terrestrial and Kongsfjorden sediments with their taxonomic affiliation and closest type strain description

Strain ID	Closest relative type strain	% similarity	Isolation source of type strain/description
Terrestrial sediments			
4VBSed**A**2	*Arthrobacter ginsengisoli (KF212463)*	99.25	From ginseng field soil, Republic of Korea
20BRSed**AA**1	*Arthrobacter pascens (X80740)*	98.54	—
20VBSed**T**4	*Cellulomonas bogoriensis (AXCZ01000188)*	96.59	From lake sediment, Kenya. An alkaliphilic, slightly halotolerant, chemo-organotrophic bacteria
4BR1Sed**T**3	*Cellulomonas cellasea (X83804)*	96.49	From soil in Japan
4VBSed**R**8	*Cryobacterium arcticum (GQ406814)*	99.4	Psychrotolerant bacteria, from soil sample from north-east Greenland
4BR1Sed**Z**5	*Cryobacterium aureum (JF267311)*	99.7	From Xinjiang No. 1 Glacier in China
4VBSed**R**7	*Cryobacterium flavum (jgi.1076268)*	99.3	From Xinjiang No. 1 Glacier in China
4BR1Sed**Z**10	*Cryobacterium levicorallinum (JF267312)*	99.85	From Xinjiang No. 1 Glacier in China
20VBSed**R**2	*Cryobacterium luteum (jgi.1076264)*	99.54	From Xinjiang No. 1 Glacier in China
4BR2Sed**Z**2	*Cryobacterium psychrotolerans (jgi.1076200)*	98.76	From Xinjiang No. 1 Glacier in China
4VBSed**R**1	*Cryobacterium roopkundense (EF467640)*	99.41	Psychrophilic bacteria from soil from glacial Lake Roopkund, Himalayan mountain range
4VBSed**T**3	*Flavobacterium degerlachei (jgi.1107974)*	99.13	From microbial mats in Antarctic lakes
20VBSed**R**5	*Flavobacterium omnivorum (AF433174)*	99.77	From Xinjiang No. 1 Glacier in China
4VBSed**AA**3	*Flavobacterium piscis (LVEN01000016)*	97.76	From diseased rainbow trout
4BR2Sed**A**3	*Flavobacterium sinopsychrotolerans (FJ654474)*	99.7	From frozen soil, China No. 1 glacier
4BR1Sed**T**4	*Herminiimonas arsenitoxidans (LT671418)*	96.97	From heavy metal-contaminated soil, oxidize arsenite
20BRSed**R**2	*Hydrogenophaga palleroni (BCTJ01000079)*	98.29	Yellow-pigmented hydrogen-oxidizing species
4BR1Sed**T**2	*Janthinobacterium lividum (Y08846)*	99.53	Common in soil and water in cold-temperate regions, produce violacein pigment, biofilm formation
4BR2Sed**Z**1	*Marisediminicola antarctica (GQ496083)*	99.78	From intertidal sediment sample from east Antarctica
4BR2Sed**T**5	*Polaromonas cryoconiti (HM583567)*	99.09	From alpine glacier cryoconite
4BR2Sed**T**2	*Polaromonas eurypsychrophila (KP013181)*	99.41	From ice core from Muztagh Glacier, Tibetan Plateau
4VBSed**T**8	*Polaromonas glacialis (HM583568)*	99.48	Psychrophilic bacteria, isolated from alpine glacier cryoconite
20BRSed**Z**4	*Polymorphobacter fuscus (KF737330)*	99.48	From permafrost soil of Kunlun mountains, Qinghai-Tibet plateau
4BR3Sed**R**8	*Pseudomonas mandelii (BDAF01000092)*	99.77	From natural mineral waters
4BR1Sed**AA**5	*Pseudomonas silesiensis (KX276592)*	97.75	From a biological pesticide sewage treatment plant
4VBSed**T**1	*Salinibacterium xinjiangense (DQ515964)*	99.7	Psychrophilic bacteria, isolated from the China No. 1 glacier
4BR1Sed**A**3	*Trichococcus collinsii (AJ306612)*	99.8	From a gas-condensate-contaminated soil in Colorado
Fjord sediments			
4KNS9Sed**R**4	*Arthrobacter humicola (AB279890)*	99.71	From paddy soil, Japan
4KNS1Sed**Z**1	*Flavobacterium degerlachei (jgi.1107974)*	99.71	From microbial mats, Antarctic lakes
4KNS1Sed**A**3	*Flavobacterium frigoris (jgi.1107976)*	99.39	From microbial mats, Antarctic lakes
20KNS1Sed**R**1	*Oceanisphaera ostreae (HQ340607)*	99.5	From seawater of an oyster farm, S.Korea
4KNS9Sed**A**1	*Paeniglutamicibacter antarcticus (AM931709)*	98.92	From Antarctic marine sediment

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Table 2 continued

Strain ID	Closest relative type strain	% similarity	Isolation source of type strain/description
4KNS9Sed**R**2	*Photobacterium frigidiphilum (AY538749)*	99.71	From deep-sea sediments in the western Pacific Ocean
4KNS9Sed**Z**1	*Planococcus halocryophilus (ANBV01000012)*	99.79	From permafrost active-layer soil from the Canadian high Arctic, growth at − 15 °C and high salinity
4KNS9Sed**Z**4	*Planomicrobium flavidum (FJ265708)*	99.28	From a marine solar saltern
4KNS9Sed**A**2	*Psychrobacter cryohalolentis (CP000323)*	99.79	From Siberian permafrost, growth at − 10 to 30 °C
4KNS1Sed**AA**3	*Psychrobacter fozii (AJ430827)*	99.64	From Antarctic coastal marine environments
4KNS1Sed**A**4	*Psychrobacter glaciei (FJ748508)*	99.86	From ice core from Austre Lovénbreen, Ny-Ålesund, Svalbard
20KNBRSed**R**2	*Psychrobacter nivimaris (AJ313425)*	100	From South Atlantic (Antarctica), particle attached bacteria
20KNBRSed**Z**3	*Paracoccuss ediminilitoris (MH491014)*	98.94	From a tidal flat sediment, East China Sea
20KNS9Sed**AA**1	*Nesterenkonia aurantiaca (HG795012)*	99.41	From a soil sample from Antarctica, haloalkaliphilic
4KNS1Sed**R**1	*Shewanella marinintestina (AB081757)*	98.01	From sea-animal intestines, ability to produce eicosapentaenoic acid

One representative isolate (having the highest base-pair length) for each species was described in the table. Sequences with similarity level < 98.7% mostly likely represent novel species are underlined. Strain IDs begin with numeric 4 or 20 representing the temperature of incubation, the alphabets written in bold indicates the media used for isolation (**A**—Antarctic Bacterial Medium, **R**—R2A Agar (1/2 strength), **Z**—Zobell Marine Agar (1/4 strength), **T**—Tryptic Soya Agar (1/10 strength), **AA**—Actinomycete Isolation Agar). For eg. **4**VBSed**A**2 is isolated from Antarctic Bacterial Medium at 4 °C incubation

concentrations of Ni, Cu, and Zn noted from the terrestrial sediments (Table 1) can be linked to the abundance of soluble rocks such as carbonates and sulfides in the glacierized areas and the contact of water with glacial debris causing the enrichment of elements (Dragon and Marciniak 2010). Łokas et al. (2016) has reported the role of atmospheric circulation transporting metals from global and local sources; marine aerosols and precipitation as major suppliers of trace metals in the Arctic glacier associated and terrestrial environments. The average values of trace metal concentrations of fjord sediments were comparable to the previously reported values from Kongsfjorden sediments (Lu et al. 2013; Grotti et al. 2017) and were lower than the baseline values reported by Lu et al. (2013). The higher concentrations of Hg and Pb towards the outer fjord were also reported by Lu et al. (2013) suggesting the influence of West Spitsbergen Current bringing relatively warm and saline waters from low and middle latitudes to the Arctic (long-range transport of metals). We could also observe significant correlations between the geochemical factors such as Ni and IC ($p < 0.005$), TOC and Hg ($p < 0.05$), Mn and Co ($p < 0.005$) as well as Hg and Pb ($p < 0.01$) (Supp Table 1). The significant correlation of TOC–Hg observed in our study may indicate the possible association of Hg to organic material (Grotti et al. 2017). Similarly, the association of Hg–Pb and Mn–Co is an indication that these metals had a similar source or may be produced by similar physicochemical processes (Bai et al. 2011).

Total microbial counts, retrievable heterotrophic bacterial counts, and phylogeny of cultivated bacterial isolates

Total microbial counts (TMC) in the sediment samples ranged between $10^{6–8}$ cells/g. The lowest count (4.3×10^6 cells/g) was observed in the terrestrial sediment BR while the highest count (4.9×10^8 cells/g) was recorded in the outer fjord sediment KNS1 (Table 1). The total microbial count observed in the terrestrial sediments was two-fold lower as compared to the counts from the fjord sediments (10^8 cells/g) in this study. Reddy et al. (2009) has reported total bacterial count in the range $2.7–11 \times 10^7$ cells/g for the sediment samples collected from the snout of Midtre Lov´enbreen glacier to the confluence point of

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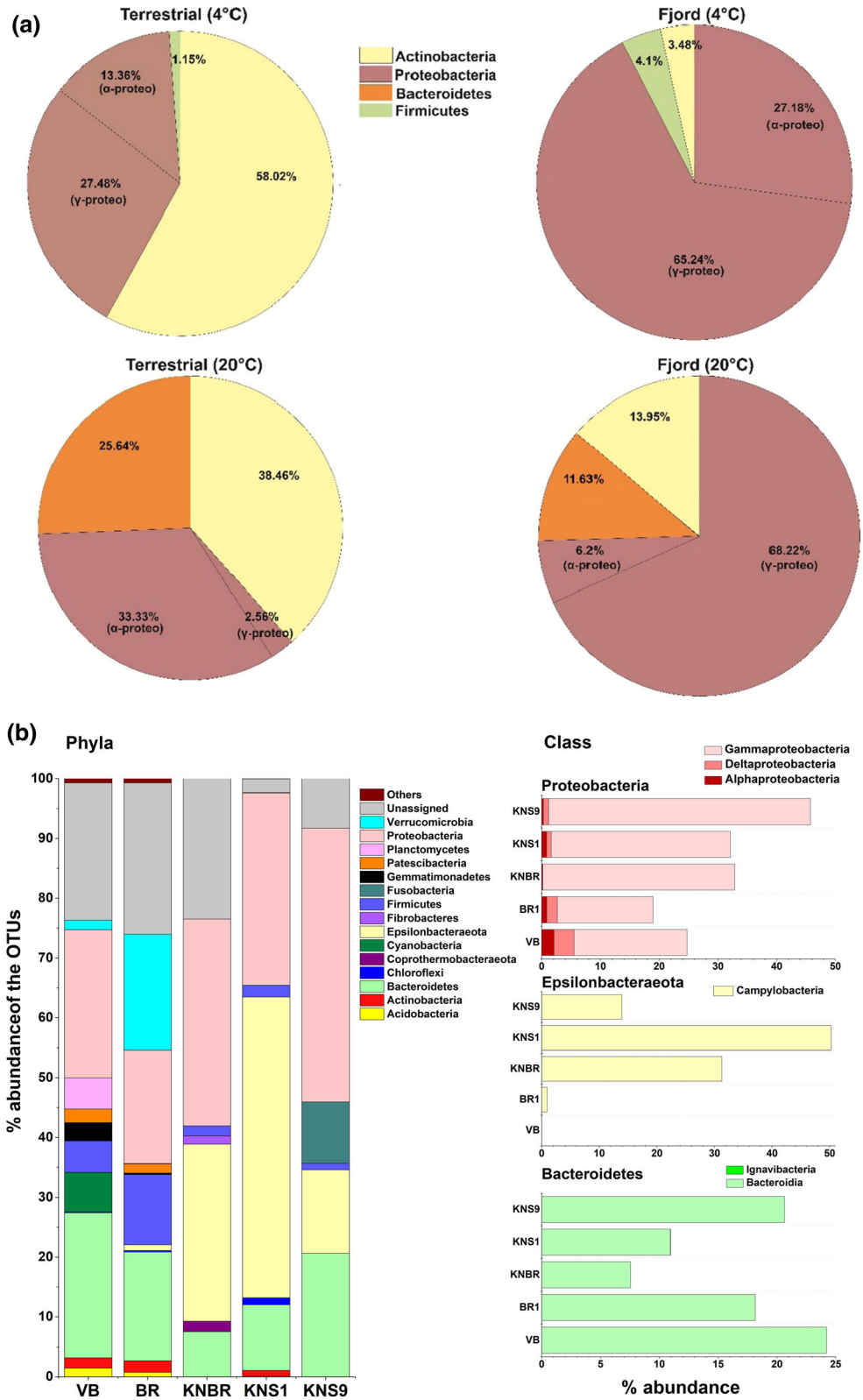

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◀**Fig. 2** **a** Percentage composition of retrievable bacteria belonging to the phylum Proteobacteria, Actinobacteria, Bacteroidetes and Firmicutes present in the terrestrial and fjord sediments from 4 °C and 20 °C incubation. Further class level abundance of the dominant phylum Proteobacteria is also shown in each pie diagram since the abundance of α-and γ-proteobacteria varied between the terrestrial and fjord sediments. **b** The relative abundance of bacterial OTUs at the phylum level and the abundance of major bacterial classes observed in the amplicon based culture-independent study

the melt water streams of the glacier with the fjord. Similarly, Conte et al. (2018) has reported the total microbial counts from the Kongsfjorden sediments to be in the range of 10^7 cells/g which is one-fold lower than the values observed in our study.

The total isolated heterotrophic bacterial count was calculated for both 4 and 20 °C incubations. Half-strength R2A media yielded the highest retrievable count for the terrestrial sediments at both the incubation temperature (4 °C: VB − 9.1×10^3 CFU/g and BR − 3.2×10^3 CFU/g and 20 °C: VB − 2.8×10^3 CFU/g and BR − 1.1×10^3 CFU/g) while quarter-strength ZMA yielded the highest number of isolates for the fjord sediments at both temperatures (4 °C: 1.6×10^5–2.5×10^5 CFU/g and 20 °C: 7.7×10^4–1.1×10^5 CFU/g) (Table 1).

106 and 64 bacterial isolates were retrieved (based on colony characteristics) from terrestrial and fjord sediments respectively from 4 °C incubation. Similarly, a total of 25 and 60 bacterial isolates were retrieved from 20 °C incubation. Pigmentation observed for more than half of the total terrestrial and fjord sediment retrievable bacterial fraction has been reported as an adaptive strategy to cold environments and for UV protection (Mueller et al. 2005). Based on the banding pattern obtained by rep-PCR, 109 bacterial isolates with unique banding patterns were selected for further identification. The results from 16S rRNA gene sequencing-based on a similarity cut-off > 98.7% revealed the presence of 27 different bacterial species in terrestrial sediments (VB and BR) and 15 in the fjord sediments (KNBR, KNS1, and KNS9). Eight bacterial sequences retrieved (7 from the terrestrial sediments and 1 from the fjord sediments) exhibited < 98.7% sequence similarity with the closest type strain in the EzBioCloud database indicating their novelty (Table 2). The dominant bacterial isolates from the terrestrial and fjord sediments were also tested to estimate their optimal temperature as well as temperature range for growth. Majority of the bacterial isolates (about 85%) were psychrotolerant (capable of growth close to 0 °C but with an optimum growth temperature of > 20 °C) while 3 isolates fit into the criteria for true psychrophiles (optimal temperature for growth at about 15 °C or lower and a maximal temperature for growth at about 20 °C). The psychrophilic isolates were closely related to the species *Polaromonas glacialis, Cryobacterium psychrotolerans* and *Polymorphobacter fuscus,* all of which were isolated from the terrestrial sediments in our study.

The bacterial isolates from the terrestrial and fjord sediments belonged to 4 phyla−Proteobacteria (constituted by class γ-proteobacteria and α-proteobacteria), Bacteroidetes, Actinobacteria, and Firmicutes (Fig. 2a). Phylum Actinobacteria dominated the terrestrial sediments at both 4 °C (58% of the total isolates) and 20 °C (38.5% of the total) incubation. The other dominant phyla isolated from the terrestrial sediments included Proteobacteria and Bacteroidetes. Bacteroidetes accounted for 13.4% of the total isolates from 4 °C and 33.3% of the total from 20 °C incubation. Class γ-proteobacteria was dominant at 4 °C (27.5% of the total) while class α-proteobacteria was dominant at 20 °C (25.6% of the total) (Fig. 2a).

Actinobacterial phyla was represented by 5 different genera, of which the genus *Cryobacterium* was dominant with 7 different species (Supp Table 2a). The class γ-proteobacteria was represented by the genera *Janthinobacterium, Herminiimonas*, *Polaromonas, Hydrogenophaga* (order Betaproteobacteriales), and *Pseudomonas* (order Pseudomonadales). The genus *Flavobacterium* solely represented the phylum Bacteroidetes while the genus *Polymorphobacte*r represented the class α-proteobacteria. The dominant species isolated from the terrestrial sediments from 4 °C incubation were *Cryobacterium psychrotolerans* (27.9% of the total species), *Janthinobacterium lividum* (19.5%), *Cryobacterium roopkundense* (19.1%), *Flavobacterium degerlachei* (7.6%), and *Polaromonas glacialis* (4.6%). Similarly, the dominant species isolated from 20 °C incubation were *Polymorphobacter fuscus* (25.6% of the total species), *Flavobacterium degerlachei* (23%), *Cryobacterium luteum* (17.9%), *Flavobacterium omnivorum* (10.3%), and *Cellulomonas cellasea* (7.7%).

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The higher abundance of bacterial isolates affiliated to the phylum Actinobacteria in the terrestrial sediments corroborated well with the studies by Edwards et al. (2013) and Zhang et al. (2016) wherein they indicated the presence of Actinobacterial members as common inhabitants of glacier forelands and cryoconites, playing a significant role in soil development and biogeochemical cycling. The three psychrophilic bacterial species isolated in our study from the terrestrial sediments are known representatives of glacier systems of Polar and high elevation environments (Zhang et al. 2007; Margesin et al. 2012; Jia et al. 2015). Of which, the genus *Cryobacterium* has been previously reported as a dominant genera associated with the supraglacier systems, known to decompose a wide variety of organic carbon substrates (Poniecka et al. 2020). Liu et al. (2020) has also observed significantly higher number of genes involved in stress response, motility, and chemotaxis in the genus *Cryobacterium* as cold adaptive survival strategies. Similar properties of carbon substrate utilization and mechanisms to withstand unfavourable conditions—through dormancy (Darcy et al. 2011) and plasmid mediated genes (Ciok et al. 2018) has been reported in the genus *Polaromonas.* The third psychrophilic species belonged to the genus *Polymorphobacter* and dominated the bacterial fraction retrieved from 20 °C incubation. *Polymorphobacter* genus comprises of all psychrotolerant members isolated from low temperature environments and known for their aerobic anoxygenic photoheterotrophic nature (Jia et al. 2015; Phurbu et al. 2020). Other dominant terrestrial isolates belonged to *Janthinobacterium* sp. which are known to produce water-insoluble purple pigment violacein as an environmental stress responsive mechanism (Pantanella et al. 2007) and *Flavobacterium sp.* known for organic matter degradation in the glacier environment (Poniecka et al. 2020; Thomas et al. 2020).

γ-proteobacteria dominated the retrievable bacterial fraction in the marine fjord sediment samples at both 4 °C (65.2% of the total) and 20 °C (68.2% of the total), with the presence of 4 genera and 6 different species (Fig. 2a). Bacterial species belonging to the phylum Actinobacteria were retrieved only from the inner fjord sediment KNS9. The dominant species retrieved from the fjord sediments at 4 °C incubation were *Psychrobacter fozii* (26.3% of the total species), *Psychrobacter cryohalolentis* (17.8%), *Psychrobacter glaciei* (16.6%), and *Flavobacterium degerlachei* (16.5%). Similarly, *Psychrobacter glaciei* (41.9%) and *Psychrobacter fozii* (13.95%) dominated the retrievable fraction at 20 °C incubation.

Higher abundance of γ-proteobacterial genus *Psychrobacter* in the fjord sediments (Supp Table 2a and b) corroborates with the previous culture-based studies from Kongsfjorden where their potential role in carbohydrate degradation, as well as cold-active enzyme production, was demonstrated (Prasad et al. 2014; Jain and Krishnan 2017; Sinha et al. 2017). The retrieval of Actinobacterial species only from the inner fjord sediments might suggest the association of Actinobacterial taxa with the cold and less saline environment.

Flavobacterium degerlachei, belonging to the phylum Bacteroidetes, was the only common representative retrieved from both the terrestrial and fjord sediments from both incubation temperatures (Table 2) suggesting their adaptability to both the terrestrial and fjord environments and corresponds well with our previous report from the meltwaters and fjord waters (Thomas et al. 2020). The versatile nature of the *Flavobacterium* species could be linked to their ability to produce poly-unsaturated fatty acids, cold-adapted enzymes, and pigments along with their specialized role in uptake and degradation of high molecular weight fraction of organic matter (Liu et al. 2019).

Bacterial diversity as revealed by 16S rRNA gene amplicon sequencing

A total of 223,613 sequences and 2376 OTUs were obtained in the present study using SILVA_132 database at 99% sequence similarity. The Good's coverage estimator of the OTUs ranged between 99.7 and 99.9% (Table 3), indicating sufficient sequence coverage in all the samples. It was found that 30,914 sequences representing 1263 OTUs were not identified based on the user database and hence have been characterized as unassigned OTUs. The unassigned OTUs accounted for about 19.98% of the total sequences identified from sediments of VB, 25% of BR, 26.8% of KNBR, 2.3% of KNS1, and 8.3% of KNS9. 1103 identified bacterial OTUs were classified into 22 phyla, 42 classes, 74 orders, 119 families, and 142 genera. In total, Proteobacteria (avg 31.2%), Epsilonbacteraeota (avg 18.9%) and Bacteroidetes (avg 16.3%) dominated the overall bacterial

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Table 3 List of total sequences, OTUs, rare taxa and various diversity indices for each sediment sample

Sample	Total sequences	Total OTUs	Goods coverage	Shannon	Simpson	Chao1
VB	92,598	1150	0.999	8.135	0.988	1244.721
BR	18,233	612	0.997	7.935	0.990	638.775
KNBR	9458	150	0.999	5.376	0.941	150
KNS1	50,046	302	0.999	5.296	0.944	322.539
KNS9	50,056	283	0.999	4.511	0.866	298.545

community. A total of 10 archaeal OTUs (1611 sequences) were identified representing the classes Bathyarchaeia, Thermoplasmata, and Nitrososphaeria. However, the number of archaeal OTUs detected in the amplicon analysis was only 0.42% of the total OTUs recovered.

The relative abundance of the major (> 1% abundance), minor (0.1–1% abundance), and rare bacterial genera (< 0.1% abundance) are summarized in Supp Fig. 2. OTUs from Proteobacteria (18.9–45.75%) and Bacteroidetes (7.5–24.2%) dominated both the terrestrial and fjord sediments. Verrucomicrobia were mostly abundant in the terrestrial sediment BR (19.4%) while Cyanobacterial OTUs were only found in the VB sediment (6.6%) Similarly, Patescibacteria and Planctomycetes were detected only in terrestrial sediments (Fig. 2b). Epsilonbacteraeota mainly resided in the outer fjord sediment KNS1 (50%) while Fusobacterial OTUs were observed only at station KNS9 (10.3%) (Fig. 2b).

The dominance of sequences affiliated to Proteobacteria, Bacteroidetes, and Verrucomicrobia from VB and BR sediments were previously reported for Svalbard lake sediments (Wang et al. 2016), although a comparatively lesser abundance of Acidobacterial phylum was found in the present study. The abundance and exclusive presence of cyanobacterial OTUs in the snout sediment (VB) might indicate their export from the supra-glacier systems to the snout region as cyanobacterial members are known to dominate the supraglacial ecosystems such as cryoconites playing a significant role in microbial community interactions and involved in cryoconite granule formation (Anesio et al. 2017; Garcia-Lopez and Cid 2017). The dominance of Verrucomicrobial OTUs in the forefield sediment BR is consistent with the results of Bendia et al. (2018), wherein they suggested the role of Verrucomicrobia in methane cycling in the glacier environments. Significant correlations were observed (Supp Table 1) between the bacterial classes Oxyphotobacteria (Cyanobacteria), VadinHA49 (Planctomycetes), ABY1 (Patescibacteria), and α-proteobacteria, which are dominant in terrestrial sediments suggesting their possible interactions with each other in the terrestrial system, contributing to ecosystem functioning. Such links were reported by Kosek et al. (2019) from Revelva catchment waters, wherein the abundance of Planctomycetes and α-proteobacteria in areas covered by cyanobacterial mats was related to their role in the degradation of algal matter. Further, the glacier snout sediment VB harbored highly diverse rare taxa (bacterial taxa having abundance < 0.1%) as compared to the other samples (64.8% of total rare taxa) (Supp Fig. 2), which in turn points out the importance of snout ecosystem acting as a 'seed bank' with less abundant highly diverse dormant taxa having unknown functional potential that could become numerically dominant and active with favorable environmental conditions (Dawson et al. 2017). This observation was further supported by the presence of 5 potential novel bacterial species from the retrievable fraction from the VB sediment (Table 2) also indicating the snout sediment to be a hotspot of rare taxa. The culture-dependent and culture-independent analysis of the snout and forefield sediments clearly indicated that their community comprise of both cosmopolitan taxa reported from the overlying meltwaters (Thomas et al. 2020), the supra-and subglacial systems (Stibal et al. 2012; Poniecka et al. 2020) and glacier-forefield soils (Nash et al. 2018; Malard et al. 2019) and rare taxa specific to the sediments.

The dominance of Proteobacteria (γ-proteobacteria), Epsilonbacteraeota, and Bacteroidetes observed in the Kongsfjorden sediments corresponds to the results of Zeng et al. (2017). An increased abundance of OTUs belonging to Epsilonbacteraeota was noted from the inner to the outer fjord sediment (Fig. 2b). This could be due to the influence of Atlantic water (Hamdan et al. 2013), which is more prevalent in the

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outer fjord (Cottier et al. 2005). Similarly, the dominance of OTUs belonging to class γ-proteobacteria (30.5–44.5%) and Bacteroidia (7.5–20.6%) in all fjord sediments is substantiated by our retrievable data, suggesting a vital linkage of the dominant bacterial taxa in the fjord sediments with organic matter degradation (Zeng et al. 2013).

The Shannon diversity index was highest for VB sediment (8.1) while the lowest value was noted for KNS9 sediment (4.5). We could observe a reduction in the bacterial diversity and richness from the terrestrial to the fjord sediments (Table 3). The higher Shannon diversity values for the terrestrial sediments are comparable to the values reported from soils and sediments from an Arctic lake in Svalbard (Wang et al. 2016). Despite the lower temperature and oligotrophic conditions prevailing in the glacier-influenced terrestrial environment, the higher diversity noted could be maintained by the mechanism of transient dormancy of the microbiota (Jones and Lennon 2010). The Shannon diversity values for fjord sediments are comparable to those reported by Zeng et al. (2017).

While comparing the amplicon sequencing results with the cultivable isolates, we could observe that all the species isolated in our study had their closest sequence variants among the OTUs from the amplicon results. Although cultivation-dependent techniques has the limitation of the selectivity of isolation media and culture conditions, it still stands significant in studying the physiological and metabolic potential of bacterial isolates from cold Polar environments which is otherwise not possible with the 16SrRNA amplicon sequencing approach. The combination of both these approaches has clearly described differences between the terrestrial and fjord sediments in terms of its community structure.

Bacterial community structure and their correlation with the sediment geochemical properties between the sampling sites

Canonical Correspondence Analysis showed that the total eigenvalue obtained as the summation of Axis 1 to 4 was 0.79. Axis 1 and Axis 2 accounted for 71.9% of the cumulative percentage variance observed in the relationship between species and the various environmental factors tested (Fig. 3). Among the various environmental factors tested in CCA, pore water pH showed significant influence on the species distribution (p-value < 0.005) explaining about 39% of the total variance by all variables. The significant influence of pH on the bacterial community structuring and their significant correlation with Acidobacterial and Actinobacterial OTUs (Supp Table1) was comparable to the results from the pan-Arctic survey of bacterial communities in Arctic soils by Malard et al. (2019). We could also observe a close association of IC (Inorganic Carbon) and trace metals such as Ni and Cd with the terrestrial VB bacterial community while Zn and sediment pore water pH exhibited a positive correlation with the BR community. Similarly, the close association of Pb, Hg, TOC, and water content with the fjord sediment communities was noted (Fig. 3). Therefore, our results establish a linkage between the environmental factors and bacterial community structuring associated with the terrestrial and fjord system.

Metabolic functional analysis of sediment communities and isolates

It was well indicated in our culture-dependent and culture-independent bacterial diversity analysis that the dominant members of the terrestrial and fjord sediments are known for their capability for organic carbon degradation. We could note significant differences in the community metabolic profiles between the terrestrial and fjord sediments at both 4 °C and 20 °C incubations ($p < 0.005$) (Fig. 4a and b). However, at both temperatures, the terrestrial sediment community had shown a higher affinity to utilize amino acids, amines and amides (24.8–35%) as compared to the fjord community (19–25.5%). Similarly, a higher affinity for utilization of carbohydrates and polymers was noted for the fjord sediment community irrespective of temperature incubations. It was found that all 5 sediment communities were positive for the utilization of D-mannitol, Tween 80, Tween 40, and N-acetyl-D-glucosamine (GlcNAc) at both 4 and 20 °C incubations (Fig. 4a and b). Mannitol being low-molecular mass organic osmolytes, are accumulated in the cytoplasm by cold-adapted bacteria, to lower the cytoplasmic freezing point and avoid desiccation by counteracting water loss and cell shrinkage during freezing (Collins and Margesin 2019). Feltracco et al. (2020) reported the prevalence of alcohol sugar mannitol in coarse particles in Arctic aerosol denoting it as a local biogenic source. GlcNAc

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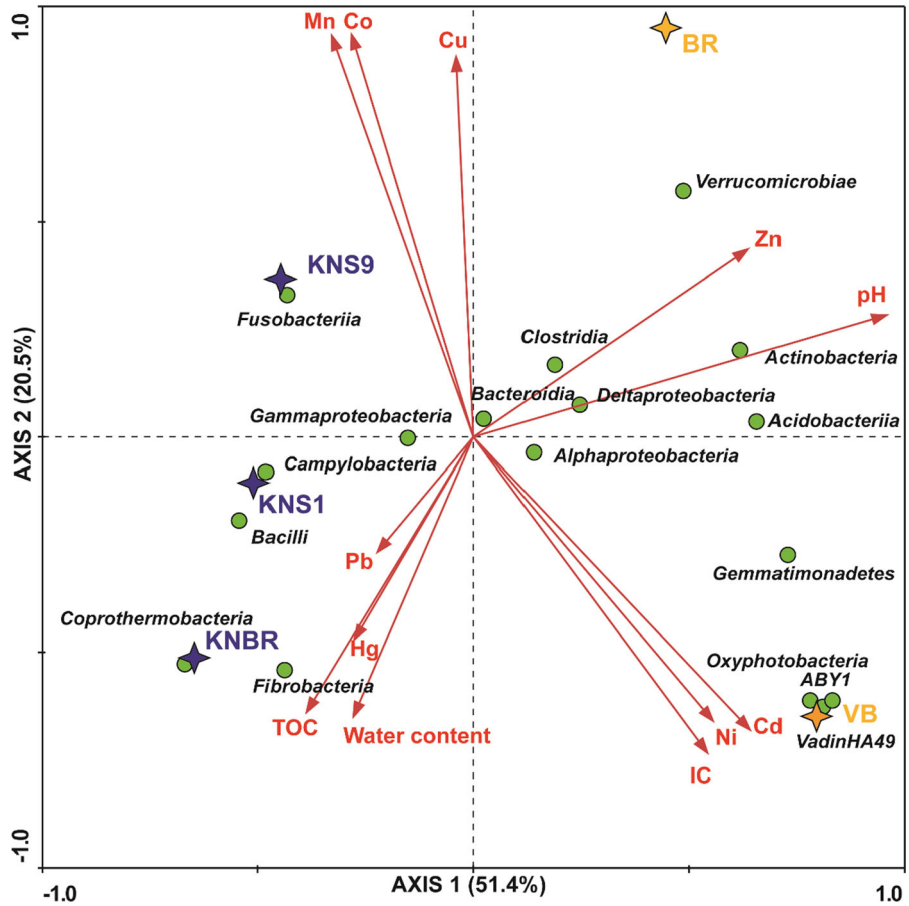

Fig. 3 Canonical correspondence analysis showing associations between the bacterial communities and geochemical properties of the terrestrial and fjord sediments. The red arrows represent environmental factors measured. The length of the arrows and the angle between each arrow and the nearest axis indicate the relative importance and closeness of that environmental factor in explaining the variation in the bacterial communities. The terrestrial sediments VB and BR are marked in orange stars and the fjord sediments KNBR, KNS1 and KNS9 are marked in blue stars

is a low-molecular weight amino sugar that is used for the synthesis of cell surface structures in bacteria and plays an important role in supplying carbon and energy by entering the glycolytic pathway after it is converted into fructose-6-phosphate (Álvarez-Añorve et al. 2005). GlcNAc uptake is reported as a widespread phenotype among marine bacteria by Cottrell and Kirchman (2000). Similarly, the Tween compounds, having an oleic acid moiety in their structure are commonly used by polar bacteria (Sala et al. 2008) and known to protect bacterial cells from adverse environmental conditions (Reitermayer et al. 2018). This is an indication that the terrestrial and fjord sediment communities utilize certain specific carbon substrates which could boost their survival mechanisms in Arctic environments.

Polymer substrates such as α-cyclodextrin and glycogen and carbohydrate substrate D-cellobiose (product of cellulose degradation), were utilized by all 3 fjord sediment communities (OD > 0.5) at both temperature incubations (Fig. 4a and b). Higher affinity for carbohydrates and complex polymer substrates over others observed in the fjord sediments suggests the presence of complex carbohydrate degrading bacterial groups in the fjord sediments (Teske et al. 2011; Jain and Krishnan 2017). Similarly, Pyruvic acid methyl ester, D-glucosaminic acid, D-galactonic acid-gamma lactone, L-arginine, L-asparagine, and L-serine were utilized efficiently by the terrestrial sediment communities (OD > 0.5) over other substrates. Significant correlations were noted between the ecoplate carbon substrates, bacterial

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community and different geochemical properties (Supp Table 3a and b). We could also observe that the linkages of community and geochemical properties with that of the ecoplate carbon substrates varied with the Ecoplate incubations at two different temperatures (Supp Table 3a and b). Shannon–Wiener (H) index and Richness index (Rs) were higher for fjord sediments as compared to terrestrial sediments at 4 °C while the values were found to be lower than terrestrial sediments at 20 °C incubation (Table 4). At these two temperatures, terrestrial and fjord communities showed variation in their metabolic responses, which may have significant implications on the substrate availability in the natural environment.

The total community metabolic response, however, might vary from the metabolic response of single bacteria representing each system. To test this hypothesis, the dominant cultivable bacterial fraction from both the terrestrial and fjord sediments was tested for their ability to grow in the presence of different carbon substrates. Some of the retrievable isolates chosen represented genera like *Flavobacterium, Polaromonas* and *Photobacterium* which accounted for $> 5\%$ relative abundance in the NGS results. For the experimental study, incubations at 4 °C and 20 °C were taken into account to determine the influence of varying temperatures on bacterial growth response. It was found that 50% of the terrestrial isolates and 70% of the fjord isolates could utilize at least 1 of the carbon substrates provided, at both temperature incubations (Table 5). Among the terrestrial isolates, the highest number of substrates were utilized by the species *Cryobacterium roopkundense* and *Arthrobacter ginsengisoli* (100% substrates utilized at 4 °C and 42% at 20 °C) belonging to Actinobacteria and *Janthinobacterium lividum* (37% and 21% of substrates utilized at 4 °C and 20 °C) belonging to Proteobacteria. Similarly, among the fjord isolates, maximum number of substrates were utilized by the species *Psychrobacter glaciei* (94.7% substrates utilized at 4 °C and 31.5% at 20 °C), *Photobacterium frigidiphilum* (47% at 4 °C and 26% at 20 °C) and *Psychrobacter nivimaris* (26% at 4 °C, 42% at 20 °C) belonging to Proteobacteria respectively (Table 5). PCA also showed that members of the bacterial phyla Proteobacteria and Actinobacteria have more affinity for utilization of different carbon substrates (Supp Fig. 3a and b).

We have also looked into the metabolic profiles of the psychrophilic species isolated in our study. Among the isolates, *Polymorphobacter fuscus* utilized carbohydrate (N-acetyl-D-glucosamine), polymer (Tween 80) and amino acid (L-serine) substrates at 4 °C while *Cryobacterium psychrotolerans* could utilize only carbohydrate substrates-D-mannitol and N-acetyl-D-glucosamine at 4 °C. Similarly, *Polaromonas glacialis* could utilize only one amino acid substrate-L-serine at 4 °C. There was no substrate utilization observed at 20 °C except for the isolate *Polymorphobacter fuscus* which could utilize Tween 80 at 20 °C (Supp Table 4a and b). Therefore, among the three psychrophilic species, *Polymorphobacter* sp. had a higher metabolic potential as compared to the other 2, although their metabolic capabilities were

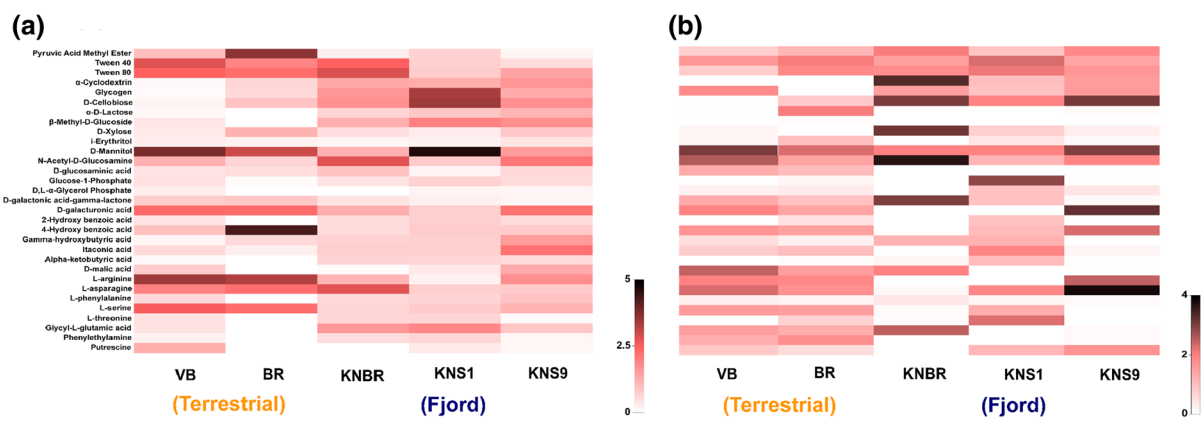

Fig. 4 **a** Heatmap of the carbon substrate utilization patterns on the Biolog Ecoplate for terrestrial and fjord sediments at 4 °C incubation and **b** Heatmap of the carbon substrate utilization patterns on the Biolog Ecoplate for terrestrial and fjord sediments at 20 °C incubation

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lower as compared to the psychrotolerant members isolated from the terrestrial system (Table 5). Comparison between the psychrophilic and psychrotolerant representatives of the genus *Cryobacterium* (*C. psychrotolerans, C. roopkundense, C. luteum*) indicated distinct metabolic capabilities for different species belonging to the same genera isolated from the same environment.

Table 4 Community level Physiological profiling results for each sediment sample incubated at 4 and 20 °C temperature incubations

Sample	AWCD		H		R	
	4 °C	20 °C	4 °C	20 °C	4 °C	20 °C
VB	0.78	1.01	2.97	2.98	22	20
BR	0.46	1.43	2.83	3.13	16	26
KNBR	0.63	0.65	3.13	2.72	26	14
KNS1	0.69	1.04	3.06	3.07	24	22
KNS9	0.67	0.67	3.21	2.70	25	14

We could also observe that at 4 °C incubation, maximum number of bacterial isolates have utilized N-acetyl-D-glucosamine > Sucrose > Tween 80 > D-mannitol = D-galacturonic acid = L-serine = L-glutamic acid > Cellulose = D-cellobiose. Similarly, at 20 °C, we found maximum utilization of Tween80 > D-mannitol > D-galacturonic acid > N-acetyl-D-glucosamine > D-cellobiose > D-glucose = D-lactose. This corroborates well with the Ecoplate results wherein substrates such as N-acetyl-D-glucosamine, D-mannitol, and Tween 80 were utilized by all the sediment communities irrespective of 4 °C and 20 °C incubations suggesting the ecological importance of these substrates in the Arctic sedimentary environments.

Similar to the community metabolic response, we could note significant differences in the bacterial growth yields (in terms of total cell count) towards different carbon substrates between the terrestrial and

Table 5 List of retrieved bacterial isolates selected for the experiment with their temperature range and number of substrates utilized for growth at 4 °C and 20 °C incubation

Bacterial Isolates chosen	Temperature optimum and range	No. of substrates utilized	
		4 °C	20 °C
Terrestrial			
Flavobacterium degerlachei (4VBSedT3)	15–20 °C (4–25 °C)	3	0
Arthrobacter ginsengisoli (4VBSedA2)	20 °C (4–35 °C)	19	8
Janthinobacterium lividum (4BR1SedT2)	15 °C (4–30 °C)	7	4
Polymorphobacter fuscus (20BRSedZ4)	10 °C (4–15 °C)	3	1
Cryobacterium roopkundense (4VBSedR1)	20 °C (4–35 °C)	19	8
Cryobacterium luteum (20VBSedR2)	25 °C (4–30 °C)	2	0
Cryobacterium psychrotolerans (4BR2SedZ2)	10 °C (4–15 °C)	2	0
Flavobacterium omnivorum (20VBSedR5)	15 °C (4–20 °C)	2	1
Polaromonas glacialis (4VBSedT8)	10 °C (4–20 °C)	1	0
Cellulomonas cellasea (4BR1SedT3)	15 °C (10–20 °C)	0	0
Fjord			
Flavobacterium degerlachei (4KNS1SedZ1)	15 °C (4–30 °C)	4	3
Psychrobacter glaciei (4KNS1SedA4)	30 °C (4–35 °C)	18	6
Paracoccus sediminilitoris (20KNBRSedZ3)	10 °C (4–25 °C)	12	0
Planococcus halocryophilus (4KNS9SedZ1)	25 °C (4–35 °C)	0	8
Psychrobacter fozii (4KNS1SedAA3)	25 °C (4–35 °C)	4	3
Photobacterium frigidiphilum (4KNS9SedR2)	25 °C (4–35 °C)	9	5
Paeniglutamicibacter antarcticus (4KNS9SedA1)	20 °C (4–35 °C)	2	3
Psychrobacter nivimaris (20KNBRSedR2)	20 °C (4–35 °C)	5	8
Psychrobacter cryohalolentis (4KNS9SedA2)	15 °C (4–20 °C)	0	0
Flavobacterium frigoris (4KNS1SedA3)	15 °C (4–30 °C)	4	1

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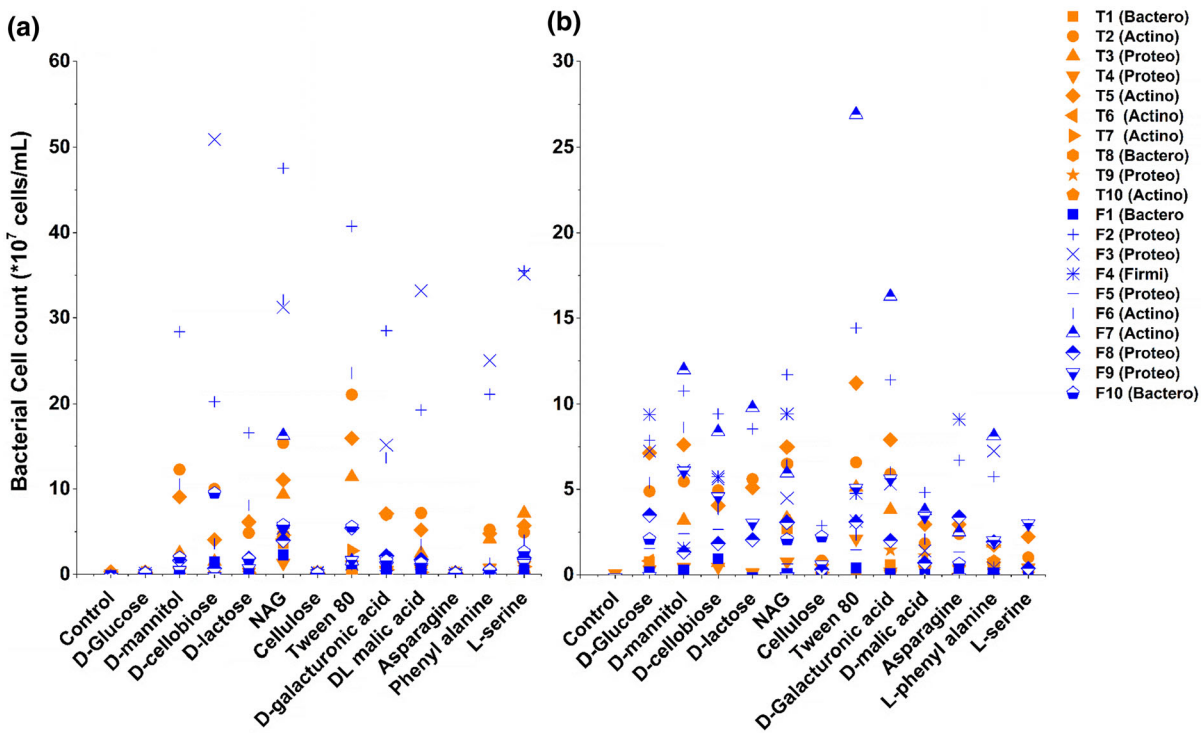

Fig. 5 **a** Bacterial growth yield results expressed in terms of bacterial cell count (*10^7cells/mL) for both terrestrial and fjord isolates grown in the presence of selected carbon substrates at 4 °C incubation observed at 12th day of growth, **b** Bacterial growth yield results for terrestrial and fjord isolates grown in the presence of selected carbon substrates at 20 °C incubation observed at 12th day of growth. Terrestrial isolates are marked as T1–T10 while fjord isolates are marked as F1–F10 along with the respective phylum details described in brackets as Bactero-Bacteroidetes, Actino-Actinobacteria, Proteo-Proteobacteria and Firmi-Firmicutes. Details of the isolates: T1-*Flavobacterium degerlachei* (4VBSedT3), T2-*Arthrobacter ginsegisoli* (4VBSedA2), T3-*Janthinobacterium lividum* (4BR1SedT2), T4-*Polymorphobacter fuscus* (20BRSedZ4), T5-*Cryobacterium roopkundense* (4VBSedR1), T6-*Cryobacterium luteum* (20VBSedR2), T7-*Cryobacterium psychrotolerans* (4BR2SedZ2), T8-*Flavobacterium omnivorum* (20VBSedR5), T9-*Polaromonas glacialis* (4VBSedT8) and T10-*Cellulomonas cellasea* (4BR1SedT3). F1-*Flavobacterium degerlachei* (4KNS1SedZ1), F2-*Psychrobacter glaciei* (4KNS1SedA4), F3-*Paracoccus sediminilitoris* (20KNBRSedZ3), F4-*Planococcus halocryophilus* (4KNS9SedZ1), F5-*Psychrobacter fozii* (4KNS1SedAA3), F6-*Photobacterium frigidiphilum* (4KNS9SedR2), F7-*Paeniglutamicibacter antarcticus* (4KNS9SedA1), F8-*Psychrobacter nivimaris* (20KNBRSedR2), F9-*Psychrobacter cryohalolentis* (4KNS9SedA2), F10-*Flavobacterium frigoris* (4KNS1SedA3)

fjord isolates at both 4 °C and 20 °C incubations (p value < 0.05). Figure 5a and b indicate a decline in bacterial growth in terms of total cell count (*10^7 - cells/ml) towards different carbon sources at 20 °C incubation as compared to 4 °C incubation. An exception was noted for the isolates F4 (*Planococcus halocryophilus*) and F7 (*Paeniglutamicibacter antarcticus*), wherein they yielded higher cell counts at 20 °C in the presence of different carbohydrates, polymers, and amino acid substrates. There was a noticeable temperature selection particularly for the terrestrial isolates i.e. 4 °C incubation found to have higher growth yields for different substrates than 20 °C incubation. Production of cold-adapted enzymes having high level of specific activity at low temperatures might have enhanced their affinity to utilize different substrates at lower temperature (Struvay and Feller 2012). This needs further evidence through experiments involving varying temperatures and varying substrate concentrations as well as whole-genome based studies on the psychrotolerant isolates which can shed light on the different mechanisms involved in high metabolic activity at temperatures much below their temperature optima for growth.

Another aspect of our study was to check whether phylogenetically related bacterial members exhibit

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similar growth response to varying substrates and varying temperature incubations or whether they are influenced by the sediment-type. For this, isolates belonging to the species *Flavobacterium degerlachei,* one retrieved from the terrestrial sediment and one from the fjord sediment were included in the study. Similarly, different bacterial species belonging to the same genera were also considered for the experiment (*Cryobacterium roopkundense, Cryobacterium luteum,* and *Cryobacterium psychrotolerans* from the terrestrial sediments, *Psychrobacter glaciei, Psychrobacter fozii, Psychrobacter nivimaris* and *Psychrobacter cryohalolentis* from the fjord sediments). Our results indicated a clear distinction in the growth response of different isolates irrespective of their phylogenetic relatedness (Table 5 and Supp Table 4a, b). Similar results were reported by Poniecka et al. (2020) wherein they observed striking differences in the metabolic capabilities of closely related isolates assigned to the same OTU. Since bacterial strains/species can adjust their substrate utilization profiles according to condition changes, the phylogenetic origin seems to be a less important structuring component of sediment bacterial communities (Freese et al. 2010). Detailed whole-genome level studies could further give insights into the metabolic pathways in phylogenetically similar bacterial species from terrestrial and fjord environments.

Conclusion

Significant differences in the carbon substrate utilization profiles between the terrestrial and fjord sediments at both 4 °C and 20 °C incubations ($p < 0.005$) implies the adaptive responses of bacterial members to utilize the available substrates in the varying natural environment. The dominance of class γ-proteobacteria observed in the fjord sediments along with the preferential utilization of carbohydrates and complex polymers in the metabolic profile of bulk sediments indicated their potential role in complex organic matter degradation in the fjord sediments. Higher taxonomic diversity with the presence of diverse rare taxa noted in the terrestrial sediment VB along with the higher affinity to utilize amino acids, amines and amides need to be emphasized further for a better understanding of their ecological role in the glacier-influenced terrestrial environment. Utilization of D-mannitol, N-acetyl-D-glucosamine, and Tween 80 by both terrestrial and fjord sediment communities as well as by the cultivated bacterial fraction indicates the readily available nature of such carbon substrates in the Arctic sedimentary environments, promoting survivability of bacteria in the extremely cold environments. The significant influence of temperature on the growth response of terrestrial and fjord isolates towards different carbon substrates was indicated in our study, with phylogeny found to be a less important structuring component of bacterial metabolic potential. Thus, our polyphasic approach contributes to a better understanding of the taxonomic and metabolic diversity of bacteria associated with the terrestrial and fjord sedimentary system, which could be a base to understand bacterial-environmental interactions and underlying ecological processes.

Acknowledgements The authors wish to express their gratitude to the Director, National Centre for Polar and Ocean Research, Ministry of Earth Sciences, India for his support and interest in this work. Thanks are due to colleagues at the Cryobiology laboratory at NCPOR, School of Environmental Sciences at M.G.University, and Kerala University of Fisheries and Ocean Studies for their support. This is NCPOR contribution number- J-13/2021-22.

Author contributions FAT and KPK designed the research plan and methodology. The formal analysis was carried out by FAT and MM. Bioinformatics analysis and statistical analysis of data were done by FAT. FAT wrote the manuscript under the supervision of KPK. All authors read and approved the manuscript.

Funding Ministry of Earth Sciences, India.

Data availability The raw nucleotide sequences generated for this study can be found in the NCBI Sequence Read Archive (SRA) database under the SRA accession number: PRJNA475645. The 16S rRNA gene sequences (104 sequences) obtained from the cultivation-dependent study are available in GenBank under the accession numbers MN080149-MN080222, MT309496-MT309525.

Declarations

Conflict of interest The authors declare no conflict of interest.

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INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY

TAXONOMIC DESCRIPTION

Thomas *et al.*, *Int. J. Syst. Evol. Microbiol.*

DOI 10.1099/ijsem.0.005375

MICROBIOLOGY SOCIETY

AQ1 *Phenylobacterium glaciei* sp. nov., isolated from Vestrebroggerbreen, a valley glacier in Svalbard, Arctic

AQ2 Femi Anna Thomas[1,2], Rupesh Kumar Sinha[1], A. A. Mohamed Hatha[3,4] and K. P. Krishnan[1,3,*]

Abstract

A Gram-stain-negative, rod-shaped bacterial strain designated as 20VBR1^T was isolated from a valley glacier (Vestrebroggerbreen) snout ice sample from Ny-Ålesund, Svalbard, Arctic. The colonies were smooth, circular and light creamish on half-strength R2A agar and grew at 10–35 °C (optimum, 20 °C), at pH 6.5–8.0 (optimum, 7.0) and with 0–2.5% (w/v) NaCl (optimum, 0.5%). 16S rRNA gene sequence analysis revealed that strain 20VBR1^T belonged to the genus *Phenylobacterium* and was most closely affiliated to *Phenylobacterium aquaticum* W2-3-4^T (97.65% similarity), *Phenylobacterium haematophilum* LMG 11050^T (97.57%) and *Phenylobacterium koreense* Slu-01^T (96.91%). 20VBR1^T has a genome size of 4.24 Mb, comprising 4185 predicted genes with a DNA G+C content of 67.86%. DNA–DNA hybridization experiments indicated that the DNA–DNA relatedness between strain 20VBR1^T and *P. aquaticum* KACC 18306^T was 41.95±4.36%, well below the threshold (<70%) to delineate bacterial species. Genome relatedness indexes revealed that the average nucleotide identity and digital DNA–DNA hybridization values between 20VBR1^T and its closest phylogenomic relative, *P. aquaticum* KACC 18306^T, were 78.97 and 22.10%, respectively. The predominant isoprenoid quinone was ubiquinone (Q-10) and the major polar lipids were phosphatidylglycerol, one unknown phospholipid, one unknown glycolipid and four unidentified polar lipids. The major fatty acids (>10 %) of strain 20VBR1^T were summed feature 8 (comprising $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$), summed feature 3 (comprising $C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$) and $C_{16:0}$. Based on the physiological, biochemical, chemotaxonomic, phylogenetic and phylogenomic analyses, isolate 20VBR1^T is considered to represent a novel species of the genus *Phenylobacterium*, for which the name *Phenylobacterium glaciei* sp. nov. is proposed. The type strain is 20VBR1^T (=JCM 33227 ^{T}=DSM 111428 ^{T}=MCC 4220 ^{T}).

The genus *Phenylobacterium,* belonging to the family *Caulobacteraceae* and class *Alphaproteobacteria*, was proposed by Lingens *et al.* [1] and the type species is *Phenylobacterium immobile*. Species belonging to the genus *Phenylobacterium* are characterized as aerobic or facultatively anaerobic, Gram-negative, motile or non-motile, straight to slightly curved rods, coccobacilli or cocci, occurring either singly, in pairs or in short chains. At the time of writing, the genus *Phenylobacterium* comprises 16 validly published species (https://lpsn.dsmz.de/genus/phenylobacterium) from diverse environments such as soil [1–5], water [6–8], compost [9], sludge [10, 11], rotating biological contactor [12], human blood [12], water purifier [13] and rhizosphere [14]. The unique chemotaxonomic features of the genus *Phenylobacterium* are ubiquinone-10 (Q-10) as the predominant isoprenoid quinone, $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$ as the major fatty acids, and phosphatidylglycerol as the major polar lipid. The DNA G+C content ranges from 64 to 72.3 mol%.

During the course of a study on the bacterial diversity associated with a glacio-marine system in Ny-Ålesund, Svalbard, Arctic, we isolated a novel bacterial strain, designated as 20VBR1^T, from ice samples collected from the snout region of Vestrebroggerbreen glacier, which is a land-terminating polythermal glacier in Ny-Ålesund, Arctic (78° 54′ 49″ N 11° 46′ 14″ E). Standard dilution

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Keywords: *Phenylobacterium*; valley glacier; snout ice; Svalbard; Arctic.
Abbreviations: ANI, Average Nucleotide Index; dDDH, digital DNA–DNA hybridization; GBDP, Genome Blast Distance Phylogeny; ML, maximum-likelihood; MP, maximum-parsimony; NCMR, National Centre for Microbial Resource; NJ, neighbour-joining.
The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Phenylobacterium* sp. 20VBR1^T is MW465305. The draft genome sequences of *Phenylobacterium* sp. 20VBR1^T and *Phenylobacterium aquaticum* KACC 18306^T were deposited in the DDBJ/EMBL/GenBank database under accession numbers JAGSGD000000000 and JALDPT000000000.
One supplementary figure and one supplementary table are available with the online version of this article.

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plating on half-strength R2A broth with agar (M1687; HiMedia; full strength contains 3.12 g per litre distilled water) at 20 °C was used for isolation. Strain 20VBR1^T was cultured continually on half-strength R2A agar at 20 °C and preserved as glycerol suspensions with 40% (w/v) glycerol at −80 °C.

16S rRNA gene sequence similarities revealed that strain 20VBR1^T has a similarity threshold below 98.7% for species demarcation and exhibited highest phylogenetic relatedness to *Phenylobacterium aquaticum* W2-3-4^T (97.65%). Therefore, we sought to ascertain the exact taxonomic status of strain 20VBR1^T following a detailed biochemical, phenotypic, chemotaxonomic, phylogenetic and phylogenomic characterization.

Cell biomass of 20VBR1^T for DNA extraction was obtained from cultures grown for 2 weeks in half-strength R2A medium at 20 °C. Genomic DNA extracted using a ChargeSwitch gDNA mini bacteria kit (Invitrogen) was used for 16S rRNA gene amplification by PCR using universal primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1510R (5′-GGTTACCTTGTTACGACTT-3′). The amplified product was further sequenced as per Sinha *et al.* [15]. A nearly complete (1361 bp) sequence of the 16S rRNA gene of strain 20VBR1^T was chimera-checked using Pintail 1.1 software and was compared with the gene sequences of closely related taxa obtained from the EzTaxon-e server [16]. Phylogenetic trees were reconstructed using the maximum-likelihood (ML) [17], maximum-parsimony (MP) [18] and neighbour-joining (NJ) [19] methods with the MEGA 7 program package [20] after multiple alignments using CLUSTAL W [21] with 16S rRNA gene sequences of all reported species of the genus *Phenylobacterium,* species from the closely related genus *Caulobacter* and the type species of *Brevundimonas*, *Asticcacaulis, Aquidulcibacter* and *Terricaulis*. Evolutionary distances were calculated according to the algorithm of Kimura's two-parameter model [22] for the ML method. The topologies of the trees were evaluated by using the bootstrap resampling method based on 1000 replications. Sequence similarity calculations using the EzTaxon-e server indicated that the closest relatives of strain 20VBR1^T were *Phenylobacterium aquaticum* W2-3-4^T (97.65%), *Phenylobacterium haematophilum* LMG 11050^T (97.57%) and *Phenylobacterium koreense* Slu-01^T (96.91%). Phylogenetic analysis based on the ML, MP and NJ (Fig. 1) methods showed that 20VBR1^T clustered with *P. aquaticum* W2-3-4^T. The 16S rRNA gene marker nucleotides of the genus *Phenylobacterium* as described in Abraham *et al.* [12] were used to determine the signature nucleotides in strain 20VBR1^T as compared with the type strains of the closest related species, *P. aquaticum* W2-3-4^T, *P. haematophilum* LMG 11050^T and *P. koreense* Slu-01^T (Table S1, available in the online version of this article). 20VBR1^T and all species compared were missing nucleotides 71–88, 183–190, 206–211 and 452–476 (*Escherichia coli* numbering). It was also observed that 20VBR1^T has 1265 A and 1270 T as compared to the closest type strains, which were found to have 1265 T and 1270 A (Table S1). 1265 A and 1270 T were also noted for *P. falsum* AC-49^T and *P. panacis* DCY 109^T among all recognized species of *Phenylobacterium*.

The reference strain *P. aquaticum* W2-3-4^T, reported by Jo *et al.* [13] as isolated from the reservoir of a water purifier, was obtained from the Korean Agricultural Culture Collection (culture collection strain ID: KACC 18306^T). The phenotypic characteristics of strain 20VBR1^T and *P. aquaticum* KACC 18306^T were determined under the same experimental conditions. Analyses of cell morphology, Gram reaction, ability to grow anaerobically, hydrolysis of various carbon sources and susceptibility to antibiotics were carried out as per Park *et al.* [23]. Scanning electron microscopy (JSM 6360LV; Jeol) was performed on exponentially growing cultures on half-strength R2A agar to examine cell morphology (Fig. 2) and motility testing was done by the hanging-drop technique [24]. Cell growth at different temperatures (4, 10, 15, 20, 25, 30, 35, 40 and 45 °C), pH (5.0–10.0 in 0.5 pH unit intervals) and NaCl concentrations (0–4% at 0.5% increments, w/v) was assessed using half-strength R2A broth. Catalase and oxidase activities were tested based on the protocol detailed by Lanyi [25]. Hydrolysis of starch (0.5% w/v), gelatin (1% w/v), carboxymethylcellulose (0.5% w/v), chitin (1%) and alginate (0.5%) was tested as detailed by Smibert and Krieg [26]. Tests for the hydrolysis of casein (skimmed milk agar), tributyrin (spirit blue agar), lecithin (egg yolk agar), DNA (DNase agar) and L-tyrosine (tyrosine agar) were carried out according to the manufacturer's protocol (HiMedia). Agar hydrolysis was determined as per Leon *et al.* [27]. Carbon substrate utilization assays were carried out using different carbon sources such as filter-sterilized sugars (0.2%, w/v), alcohols (0.2%, v/v), organic acids (0.1%, w/v) or amino acids (0.1%, w/v) in the basal medium as per Farmer *et al.* [28]. A Gram-negative MicroPlate (GN2; BIOLOG) was used to report the metabolic profile of strain 20VBR1^T following the manufacturer's instructions. For this, a 2-week-old culture (20VBR1^T) was grown on half-strength R2A agar at the optimum growth temperature. Additional enzyme activities, including nitrate reduction, were tested using API ZYM and API 20NE kits (bioMérieux) as per the manufacturer's instructions. Antibiotic sensitivity was assessed using the disc diffusion agar method [29]. The antibiotics (µg per disc) used were: cefotaxime (30), neomycin (30), erythromycin (15), nitrofurantoin (300), nalidixic acid (30), polymyxin B (300), rifampicin (30), streptomycin (10), tobramycin (10), kanamycin (30), carbenicillin (100), trimethoprim (5), amoxicillin (25), tetracycline (30), novobiocin (30), penicillin G (10), gentamycin (10), chloramphenicol (30), ampicillin (10) and vancomycin (30).

Cells of strain 20VBR1^T were Gram-stain-negative, aerobic, non-spore-forming, non-motile and long rod-shaped (measuring 0.4–0.5×1.0–2.0 µm). Colonies grown on half-strength R2A agar plates for 2 weeks at 20 °C were smooth, circular, convex, light creamish and 0.5–1 mm in diameter. The morphological, physiological and biochemical characteristics of strain 20VBR1^T are given in the species description and Table 1, which lists the characteristics that serve to differentiate 20VBR1^T from its closest phylogenetic relatives. Strain 20VBR1^T was resistant to polymyxin-B, penicillin, ampicillin, amoxicillin, chloramphenicol and nitrofurantoin, but susceptible to rifampicin, nalidixic acid, tobramycin, carbenicillin, streptomycin, cefotaxime, tobramycin, trimethoprim, tetracycline, erythromycin, neomycin, novobiocin and vancomycin.

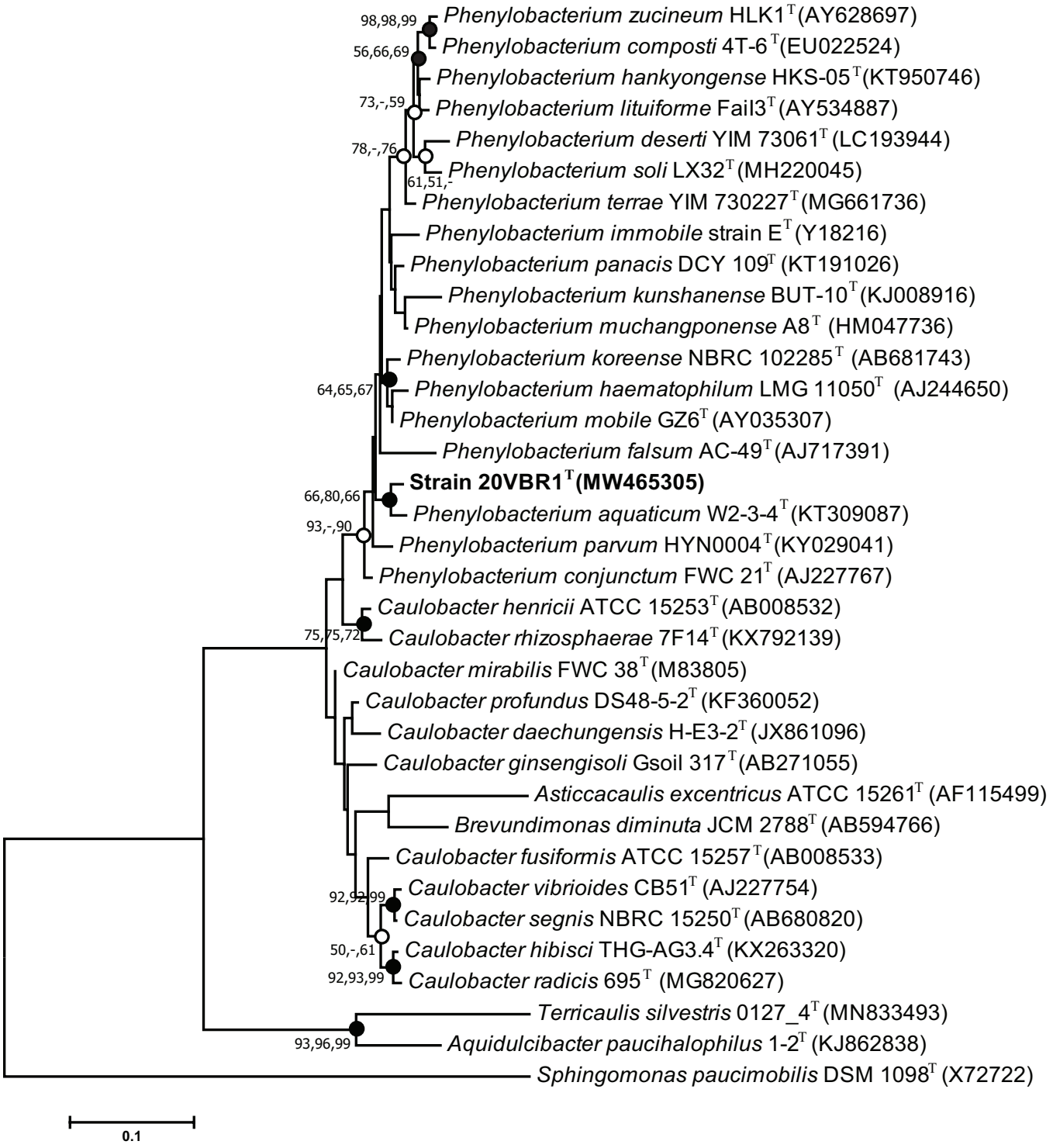

Fig. 1. Rooted ML tree showing the phylogenetic relationship between strain 20VBR1^T and validly reported species of the genus *Phenylobacterium* and closely related genera *Caulobacter*, *Brevundimonas, Asticcacaulis, Terricaulis and Aquidulcibacter* based on unambiguously aligned 16S rRNA gene sequences (954 bp). *Sphingomonas paucimobilis* DSM 1098^T was used as an outgroup. Bootstrap values are given at nodes (ML/MP/NJ). Filled circles indicate that the corresponding nodes were also recovered in the trees generated using both the MP and NJ algorithms, and open circles indicate that the nodes were recovered in the trees generated using either one of the MP or NJ methods respectively. A dash indicates no value. Bar, 0.1 nt substitutions per nucleotide position.

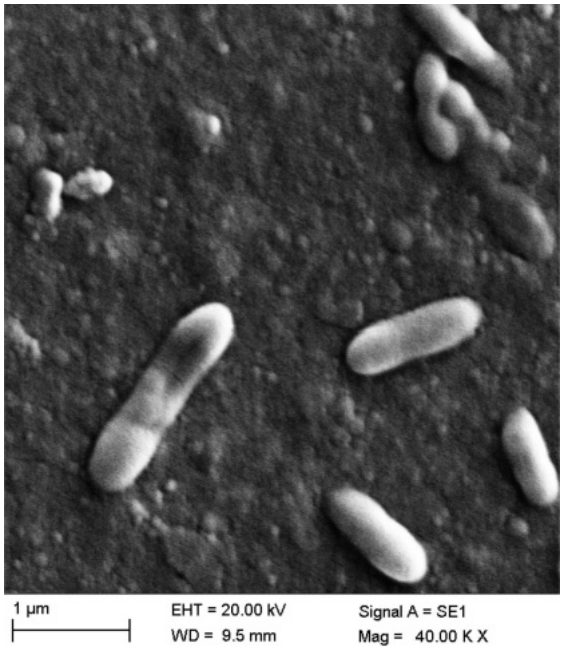

Fig. 2. Scanning electron micrograph of cells of strain 20VBR1^T grown on half-strength R2A agar at 20 °C for 2 weeks. Bar, 1 µm.

Analysis of the chemotaxonomic properties such as respiratory quinones and polar lipids for strain 20VBR1^T was done through the DSMZ-Identification Service (Germany) and National Centre for Microbial Resource (NCMR), India. In brief, extraction of respiratory quinones was carried out from 50 mg freeze dried bacterial cells using hexane followed by purification using a silica-based solid phase extraction method [30, 31]. Analysis of the purified sample using HPLC (reversed-phase column) allowed the relative quantification of ubiquinones at 270 nm and menaquinones at 326 nm. The cultures were harvested at the logarithmic growth phase and the pellet was used for polar lipid extraction with methanol/chloroform/0.3% sodium chloride (2:1:0.8, by vol.) as described by Bligh and Dyer [32] considering the modifications of Card [33]. Lipids were separated using silica gel TLC (Kieselgel 60F254; Merck) by two-dimensional chromatography using chloroform/methanol/water (65:25:4, by vol.) in the first dimension and chloroform/acetic acid/methanol/water (40:7.5:6:2, by vol.) in the second dimension [34]. The dried plates were subjected to spraying with 5% ethanolic phosphomolybdic acid for total lipids and further characterized by spraying with ninhydrin (specific for aminogroups), molybdenum blue (specific for phosphates), Dragendorff reagent (quaternary nitrogen) or α-naphthol (specific for sugars). Cellular fatty acid analysis was carried out for strain 20VBR1^T and *P. aquaticum* KACC 18306^T by Gas Chromatography, at NCMR, (GC system - 7890A; Agilent Technologies) according to the rapid Microbial Identification System software (MIS, MIDI; version 6.0, RTSBA6 database) as described by Sasser [35].

The major respiratory quinone of 20VBR1^T was Q10, which is in line with all other members of the family *Caulobacteraceae*. The lipid profile of 20VBR1^T comprised phosphatidylglycerol, which has been generally reported for other species of *Phenylobacterium*, along with an unknown phospholipid, one unknown glycolipid and four unidentified polar lipids (Fig. S1). The presence of an unknown phospholipid, unknown glycolipid and unidentified polar lipids has been reported for the closest related type strain, *P. aquaticum* W2-3-4^T [13]. The fatty acid profile of strain 20VBR1^T mainly comprised summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$, 40.25%), summed feature 3 ($C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$, 19.18%), $C_{16:0}$ (10.2%), $C_{18:1}\omega7c$ 11-methyl (6.55%) along with $C_{12:1}$ 3-OH (2%) as the major hydroxyl fatty acid. However, some quantitative differences in fatty acid content was observed for 20VBR1^T in comparison with *P. aquaticum* KACC 18306^T and the other closely related species *P. haematophilum* CCUG 26751^T and *P. koreense* Slu-01^T, such as higher values observed for summed feature 3 for strain 20VBR1^T (Table 2). Summed feature 3 accounted for about 19.18% of the fatty acid profile of 20VBR1^T as compared to *P. aquaticum* KACC 18306^T (1.88%) and this was the highest value noted among all the members of the genus *Phenylobacterium*. Similarly, unsaturated fatty acid $C_{18:1}\omega7c$ 11-methyl accounted for <10% of the total fatty acids in 20VBR1^T while the same accounted for 21.7% in *P. aquaticum* KACC 18306^T and 28% in *P. haematophilum* CCUG 26751^T [13] but was not reported in *P. koreense* Slu-01^T [10].

The whole-genome sequence of strain 20VBR1^T was determined using an Illumina HiSeq X10 (Illumina) at Agrigenome Labs, India, and Nanopore MinION long-read sequencer (Oxford Nanopore Technologies) using a sequencing ligation kit (SQK-LSK 109) on flow cell v. 9.4.1 carried out at the home laboratory at NCPOR, India, respectively. Genome assembly was performed with reads from both sequencing technologies using the hybrid assembly method of Unicycler v. 0.4.8 [36]. The assembly yielded

Table 1. Major phenotypic characteristics that differentiate 20VBR1^T from closely related type strains of the genus *Phenylobacterium*

Strains: 1, 20VBR1^T (data from this study); 2, *P. aquaticum* KACC 18306^T (data from this study, otherwise taken from Jo *et al*. (2016) [13], indicated by *); 3, *P. haematophilum* CCUG 26751^T [12, 13]; 4, *P. koreense* Slu-01^T [10]. All species were positive for activity of oxidase and acid phosphatase. All species were negative for utilization of L-arabinose, maltose and *N*-acetyl-glucosamine; and activities of arginine dihydrolase, urease, cystine arylamidase, esterase (C4), esterase lipase (C8), lipase (C14), α-galactosidase, *β*-galactosidase, *β*-glucuronidase, α-mannosidase and α-fucosidase. +, Positive; –, negative.

Characteristic	**1**	**2**	**3**	**4**
Isolation source	Glacier snout ice	Reservoir of water purifier	Human blood	Activated sludge
Cell size (μm)	0.4–0.5×1.0–2.0	0.4–0.6×2.5–4.0*	0.3–0.4×0.9–2.5	0.7–1.0×1.0–2.0
Motility	–	–	+	–
Temperature range (°C) (optimum)	10–35 (20)	18–40* (25–30)	10–40 (37)	20–37 (28–30)
pH range (optimum)	6.5–8.0 (7.0)	6.0–8.0* (7.0)	6.0–8.5	6.5–8.5 (7.0–8.0)
NaCl range (%, w/v) (optimum)	0–2.5 (0.5)	0–0.5*	0–2.0 (0.5)	0–2.0 (0)
Nitrate reduction to nitrite	+	+	–	+
Oxidase	+	+	+	+
Catalase	–	–	–	+
Utilization of:				
D-Glucose	–	–	+	–
D-Mannitol	–	–	–	–
L-Phenylalanine	–	+	–	+
L-Rhamnose	+	–	–	–
D-Mannose	+	–	–	–
API-ZYM results				
Alkaline phosphatase	+	+	+	+
Valine arylamidase	+	+	–	–
α-Chymotrypsin	–	+	–	–
β-Glucuronidase	–	+	–	–
β -Glucosidase	+	+	+	–
α-Glucosidase	+	+	–	–
DNA G+C content	67.86%	68.87%	67.9%	68.1 mol%

971× coverage with a total length of 4242328 bp. The draft genome of strain 20VBR1^T consisted of a single linear chromosome of 4.24 Mb size distributed across five contigs, of which the largest contig size was 3821543 bp. The assembled genome showed 99.03% completeness and 2.38% contamination as assessed by the bioinformatics tool CheckM [37]. Functional annotation was done using Prokka v. 1.13 [38]. The annotation predicted 4185 genes, including 4114 protein coding genes, and 50 total RNAs (three rRNAs, 43 tRNA genes and four non-coding RNAs). As the genome sequence of the closest phylogenetic neighbour *P. aquaticum* was not available, whole-genome sequence analysis was carried out for *P. aquaticum* (KACC 18306^T) using the Ion Genestudio S5 system (Ion Torrent; Thermofisher Scientific) at NCPOR, India. Ion Torrent S5 libraries were prepared using the 'Ion 540' for the Ion Chef Kit for 200 base-read libraries and sequenced on the Ion Torrent S5 using an Ion 540 semi-conductor sequencing chip (Thermo Fisher). Genome assembly was carried out using SPAdes v. 3.1.0, which yielded 751× coverage with a total length of 5245083 bp. The genome of *P. aquaticum* KACC 18306^T consisted of 90 contigs (>1000 bp), of which the largest contig size was 396914 bp. The assembled genome showed 99.55% completeness and 2.87% contamination as assessed by the bioinformatics tool CheckM [37]. The G+C content of the genomic DNA of strain 20VBR1^T was 67.86%, which is within the

Table 2. Cellular fatty acid composition (%) of strain 20VBR1^{T}and closely related species of the genus *Phenylobacterium*.

Strains: 1, 20VBR1^T (data from this study); 2, *P. aquaticum* KACC 18306^T (data from this study); 3, *P. haematophilum* CCUG 26751^T (Jo *et al*., 2016 [13]); 4, *P. koreense* Slu-01^T (Aslam *et al*., 2005 [10]). –, Not detected; TR, trace amount. Major components (>10%) are highlighted in bold.

Fatty acid	1	2	3	4
Saturated				
$C_{10:0}$	–	2.1	–	–
$C_{11:0}$	–	0.69	TR	2.1
$C_{12:0}$	–	0.3	2.3	4.0
$C_{13:0}$	–	–	–	2.0
$C_{14:0}$	0.48	0.2	TR	TR
$C_{15:0}$	6.77	–	–	9.6
$C_{16:0}$	**10.2**	**30.3**	**17.4**	**10.4**
$C_{17:0}$	4.0	**10.67**	7.6	8.7
iso-$C_{17:0}$	0.5	0.75	TR	–
$C_{18:0}$	–	3.7	TR	7.3
$C_{20:0}$	–	–	1.3	2.4
Unsaturated				
$C_{15:1}\omega 8c$	–	–	–	1.4
$C_{16:1}\omega 11c$	0.5	1.3	1.6	4.4
$C_{17:1}\omega 6c$	4.2	1.27	2.0	3.7
$C_{17:1}\omega 8c$	1.56	1.09	0.9	1.2
$C_{17:1}\omega 9c$	–	–	–	2.5
$C_{18:1}\omega 7c$	**40.25**	**16.86**	**25.4**	**23.5**
$C_{18:1}\omega 7c$ 11-methyl	6.55	**21.7**	**28**	–
Hydroxyl fatty acids				
$C_{12:0}$ 2-OH	–	–	–	–
$C_{12:0}$ 3-OH	0.79	0.85	0.8	TR
$C_{12:1}$ 3-OH	2.0	1.7	1.9	3.4
$C_{18:1}$ 2-OH	–	–	–	4.9
Cyclo fatty acids				
$C_{19:0}$ cyclo $\omega 8c$	–	0.57	2.7	
Summed features				
3 ($C_{16:1}\omega 6c$ and/or $C_{16:1}\omega 7c$)	**19.18**	1.88	5.9	–
8 ($C_{18:1}\omega 6c$ and/or $C_{18:1}\omega 7c$)	**40.25**	**16.86**	**25.4**	**23.5**

general range for members of the genus *Phenylobacterium*, and was similar to those of *P. aquaticum* KACC 18306^T (68.87%), *P. haematophilum* DSM 21793^T (67.90%) and *P. koreense* Slu-01^T (68.1 mol%) (Table 1).

The DNA–DNA relatedness between 20VBR1^T and the closest type strain *P. aquaticum* KACC 18306^T was carried out by performing DNA–DNA hybridization experiments according to the method of Marmur [39]. The analysis was carried out at NCMR by fluorimetry using a Step One Plus Real-Time PCR system (Applied Biosystems) as described by Loveland-Curtze *et al.* [40]. The reassociation of DNA was carried out at an optimum reassociation temperature 73.0 °C with 30% formamide according to De Ley *et al.* [41] and Gillis *et al.* [42]. DNA–DNA relatedness between strain 20VBR1^T and *P. aquaticum* KACC 18306^T was 41.95±4.36%, far below the recommended threshold (<70%) to delineate bacterial species as per Wayne *et al.* [43].

Table 3. dDDH values (%), orthoANIu values (%) and G+C content difference (%) between the strain 20VBR1^T genome and the reference genomes of closely related species of the genus *Phenylobacterium*

Species comparison with 20VBR1^T	dDDH (%)	orthoANIu (%)	G+C content difference (%)
P. aquaticum KACC 18306	22.1	78.97	1.01
P. haematophilum DSM 21793	21.2	77.95	0.05
P. hankyongense HKS-05	21.0	78.13	2.41
P. soli LX32	20.2	76.76	2.21
P. kunshanense BUT-10	20.2	75.87	1.39
P. zucineum HLK1	20.1	76.80	3.25
P. immobile E	20.1	75.04	1.13
P. parvum HYN0004	19.9	75.91	2.04
P. deserti YIM 73061	19.7	75.63	0.32

Average nucleotide index (ANI) and digital DNA–DNA hybridization (DDH) analysis were carried out using the genomes of the closest species belonging to the genus *Phenylobacterium*. ANI was calculated using the orthoANIu (%) calculator in the EZbiocloud server [44] (www.ezbiocloud.net/tools/ani). The orthoANIu values between strain 20VBR1^T and *P. aquaticum* KACC 18306^T and *P. haematophilum* DSM 21793^T were 78.97 and 77.95%, respectively, well below the cut-off value of 95–96% proposed for species delineation [45] (Table 3). Similarly, *in silico* digital DDH (dDDH) was carried out using the Genome to Genome Distance Calculator (http://ggdc.dsmz.de/), between strain 20VBR1^T and the genomes of other closely related species of the genus *Phenylobacterium* [46]. The dDDH values between strain 20VBR1^T and *P. aquaticum* KACC 18306^T reached 22.10% (the highest value obtained), values well below the 70% species cut-off [45, 47] (Table 3).

A genome-based phylogenetic tree was reconstructed using the Type (Strain) Genome Server provided by DSMZ [48] to further verify the phylogenetic position of strain 20VBR1^T. For the phylogenomic inference, all pairwise comparisons among the set of genomes were conducted using Genome Blast Distance Phylogeny (GBDP) approach, and accurate intergenomic distances were inferred under the algorithm 'trimming' and distance formula d5 [46]. The resulting intergenomic distances were used to infer a balanced minimum evolution tree with branch support via FastME 2.1.6.1 [49]. Branch support was inferred from 100 pseudo-bootstrap replicates each. The trees were rooted at the midpoint [50] and visualized with PhyD3 [51]. Similar to the results of the 16S rRNA gene-based phylogenetic tree, the phylogenomic tree showed strain 20VBR1^T forming a separate branch with *P. aquaticum* KACC 18306^T.

The phylogenetic (Fig. 1) and phylogenomic distance (Fig. 3) between strain 20VBR1^T and recognized *Phenylobacterium* species along with the unique phenotypic and chemotaxonomic characteristics (Tables 1 and 2) affirms the assignment of 20VBR1^T to the genus *Phenylobacterium* as the type strain of a novel species, for which the name *Phenylobacterium glaciei* sp. nov. is proposed.

DESCRIPTION OF *PHENYLOBACTERIUM GLACIEI* SP. NOV.

Phenylobacterium glaciei (gla.ci.e'i. L. gen. n. *glaciei* of ice).

Cells are Gram-stain-negative, oxidase-positive, catalase-negative, strictly aerobic, non-motile, non-spore forming and rod shaped (0.4–0.5 µm in diameter and 1.0–2.0 µm in length). After growth on half-strength R2A agar for 2 weeks, colonies appear smooth, circular, light creamish, convex and 0.5–1 mm in diameter. Grows on half-strength R2A agar at 10–35 °C (optimum, 20 °C), at pH 6.5–8.0 (optimum, 7.0) and with 0–2.5% (w/v) NaCl (optimum, 0.5%). Negative for starch, DNA, cellulose, casein, chitin, gelatin, alginate, skimmed milk and agar hydrolysis. Nitrate is reduced to nitrite. Does not assimilate L-phenylalanine but utilizes D-mannose, D-melizitose and L-rhamnose as sole carbon sources. Possesses activities of alkaline phosphatase, valine arylamidase, *β*-glucosidase and α-glucosidase, but not esterase (C4), lipase (C14), cystine arylamidase, esterase lipase (C8), trypsin, naphthol-AS-BI-phosphohydrolase, α-chymotrypsin, α-galactosidase, *β*-galactosidase, *β*-glucuronidase, *N*-acetyl-*β*-glucosaminidase, α-mannosidase, leucine arylamidase, acid phosphatase, α-fucosidase, arginine dihydrolase or urease. Positive for utilization of *cis*-aconitic acid, D,L-lactic acid, D,L-α-glycerol phosphate, citric acid, D-galactonic acid-γ-lactone, D-galacturonic acid, D-gluconic acid, D-glucuronic acid, D-saccharic acid, glucose 1-phosphate, glycerol, itaconic acid, L-histidine, *N*-acetyl-D-galactosamine, *p*-hydroxyphenyl acetic acid, succinic acid, urocanic acid and α-D-lactone. Negative for utilization of α-D-glucose, lactose, sucrose, D-galactose, maltose, raffinose, trehalose, D-mannitol, D-mannose, sorbose, ethanol, Tween 40, Tween 80, D-mannose, D-glucosaminic acid, D-glucose 6-phosphate, maltose, melibiose, D-arabitol, D,L-carnitine, cellobiose, dextrin, D-fructose, D-psicose, 2,3-butanediol, 2-aminoethanol, acetic acid, adonitol, α-ketovaleric acid, bromosuccinic acid,

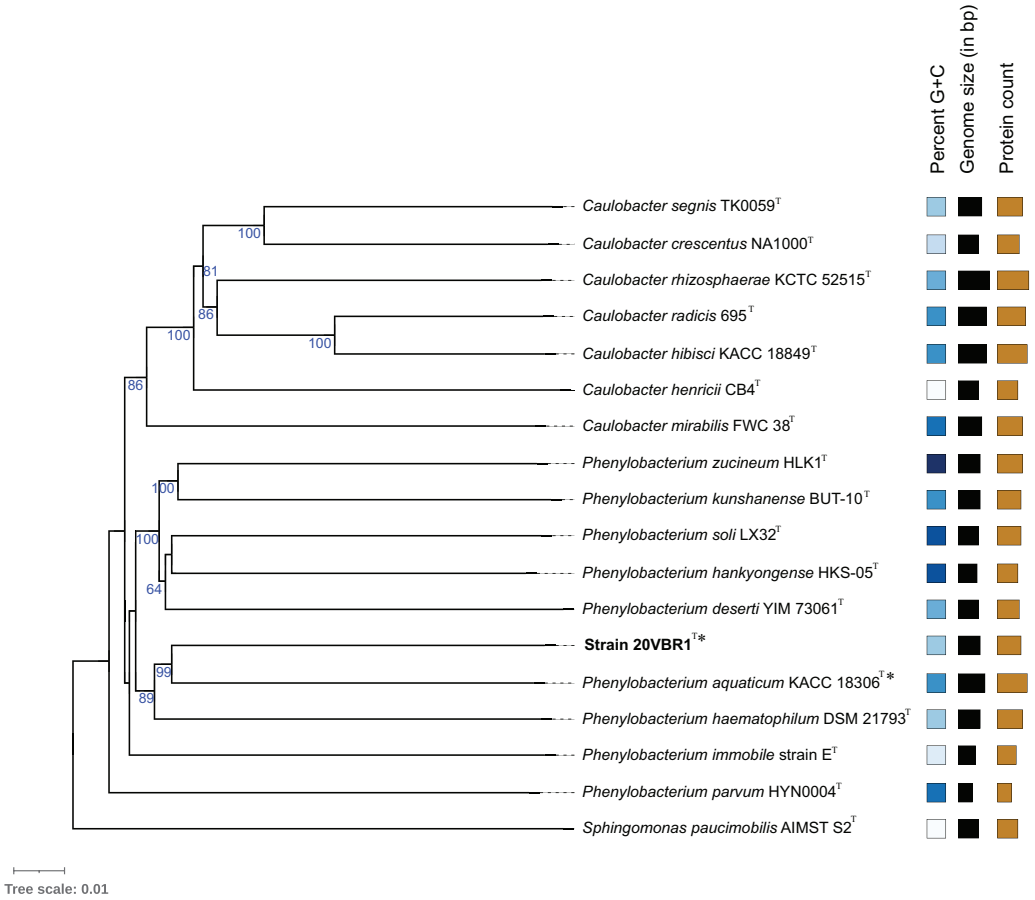

Fig. 3. Phylogenomic tree inferred with FastME 2.1.6.1 [49] from Genome Blast Distance Phylogeny (GBDP) distances calculated from genome sequences, showing the phylogenetic position of strain 20VBR1^T (https://tygs.dsmz.de). Branch lengths are scaled in terms of GBDP distance formula d5. Numbers above branches are GBDP pseudo-bootstrap support values >60% from 100 replications, with average branch support of 76.9%. The tree was rooted at the midpoint [50]. The genome sequence of *Sphingomonas paucimobilis* AIMST S2^T was used as an outgroup. *The genome sequenced in our study. Bar, 0.01 substitutions per nucleotide position.

D-alanine, D-serine, D-sorbitol, formic acid, gentiobiose, glucoronamide, glycogen, glycyl-L-aspartic acid, glycyl-L-glutamic acid, hydroxyl-L-proline, i-erythritol, inosine, L-aspartic acid, lactulose, L-alaninamide, L-alanine, L-alanyl glycine, L-arabinose, L-asparagine, L-fucose, L-glutamic acid, L-leucine, L-ornithine, L-phenyl alanine, L-proline, L-pyroglutamic acid, L-rhamnose, L-serine, L-threonine, malonic acid, *myo*-inositol, methyl pyruvate, *N*-acetyl-D-glucosamine, phenyl ethyl amine, propionic acid, putrescine, quinic acid, sebacic acid, succinamic acid, succinic acid monomethyl ester, thymidine, turanose, uridine, xylitol, α-cyclodextrin, α-hydroxybutyric acid, α-ketoglutaric acid, *β*-hydroxybutyric acid, methyl-*β*-D-glucoside, γ-aminobutyric acid and γ-hydroxybutyric acid. Q-10 is the predominant respiratory quinone and summed feature 8 (comprising $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$), summed feature 3 (comprising $C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$), $C_{16:0}$ and $C_{18:1}\omega7c$ 11-methyl are the major cellular fatty acids with $C_{12:1}$ 3-OH as the major hydroxyl fatty acid. The polar lipids are phosphatidylglycerol, an unknown phospholipid, an unknown glycolipid and four unidentified polar lipids.

The type strain, 20VBR1^T (=JCM 33227 ^{T}=DSM 111428 ^{T}=MCC 4220 ^{T}), was isolated from a glacier ice sample from Ny-Ålesund, Svalbard, Arctic. The GenBank accession numbers for the 16S rRNA gene and complete genome sequence are MW465305 and JAGSGD000000000 respectively. The G+C content of the genomic DNA of the type strain is 67.86%.

Data availability statement

The GenBank accession number for the 16S rRNA gene sequence of *Phenylobacterium* sp. 20VBR1^T is MW465305. The draft genome sequence of *Phenylobacterium* sp. 20VBR1^T and *P. aquaticum* KACC 18306^T were deposited in the DDBJ/EMBL/GenBank database under accession numbers JAGSGD000000000 and JALDPT000000000.

Funding information
Ministry of Earth Sciences, India.

Acknowledgements
The authors wish to express their gratitude to the Director, National Centre for Polar and Ocean Research, Ministry of Earth Sciences, India, for his support and interest in this work. Thanks are due to colleagues at the Cryobiology laboratory at NCPOR and CUSAT-NCPOR centre for Polar sciences for their support. This is NCPOR contribution number XXXX.

Author contributions
Conceptualization: F.A.T., K.P.K., R.K.S.; Formal analysis and investigation: F.A.T., K.P.K., R.K.S., A.A.M.H.; Writing original draft preparation: F.A.T.; Writing - review and editing : K.P.K., R.K.S., F.A.T. All authors read and approved the manuscript.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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Annexure III

Metal stock solution preparation used for the enrichment study

Heavy metals	Molarity of stock prepared	Quantity of metal salt added in Milli Q (25mL)
Hg ($HgCl_2$)	1mM	6.7875g/25mL
	100µM	2.5mL of 1mM/25mL
Pb (Pb $(NO_3)_2$	10mM	82.8mg/25mL
	1mM	2.5mL of 10mM /25mL
Cd ($CdCl_2$. H_2O)	10mM	50mg/25 mL
	1mM	2.5mL of 10mM /25mL
Ni ($NiCl_2.6H_2O$)	10mM	0.059g/25mL
	1mM	2.5mL of 10mM /25mL
Co($CoCl_2.6H_2O$)	10mM	0.059g/25mL
	1mM	2.5mL of 10mM /25mL
Zn ($ZnCl_2$)	100mM	0.34g/25mL
	10mM	2.5mL of 100mM /25mL
Mn($MnCl_2.4H_2O$)	100mM	4.94g/25mL
	10 mM	2.5mL of 100mM /25mL


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